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Physiological Zoölogy

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EXPERIMENTAL INTERSEXUALITY: THE RELATIVE SENSITIVITY OF MALE AND FEMALE RAT EMBRYOS TO ADMINISTERED ESTROGENS AND ANDROGENS*

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EVIDENCE has been presented in a previous paper (Greene, Burrill, and Ivy, 1941) that the "male" and "female" hormones are mutually antagonistic in their effects on the differentiation of most embryonic sexual structures in the rat. This evidence was obtained by administering large doses of androgens and estrogens in varying proportions to the pregnant rat and by studying the effects of these combined hormones on the newborn offspring. Estrogens, if given in sufficient amounts, nullified the usual masculinizing effects of androgens in the female embryos. Androgens, in turn, nullified the usual feminizing effects of estrogens in the male embryos.

The present communication is a further consideration of the data of the preceding study with the object of investigating a possible difference between males and females in the response of their sexual structures to the combined hormones.

PROCEDURE

Pregnant rats were injected daily, from the fourteenth to the twenty-first day of pregnancy, with an oil solution containing the combined estrogens and androgens. The animals were killed on the twenty-second day (normal day of delivery in our colony). The fetuses were removed from the uteri, examined under a dissecting microscope, and fixed in Bouin's fluid. Representative newborn were serially sectioned. The number of newborn observed, the number studied by serial section, the hormones used, and the doses administered are presented in Table 1. The groups in this table are numbered as in the previous publication. Several groups are omitted, either because there were animals of only one sex serially sectioned in the group or because the dosage was such that the findings are not relevant.

* Supported in part by grants from the Josiah Macy, Jr., Foundation and Ciba Pharmaceutical Products, Inc.

REVIEW OF HORMONAL EFFECTS ON SEXUAL DIFFERENTIATION OF RAT EMBRYOS

Before presenting and comparing the effects of the combined hormones in the male and female, it is necessary to review the findings in the newborn animals exposed to each of the hormones alone. Complete details have been presented in our previous publications (Greene, Burrill, and Ivy, 1939*a*, *b*, 1940). Inasmuch as differentiation of the gonads is not modified by these procedures, they will not be mentioned further.

A. ANDROGENS

Androgens given to the pregnant rat have no effect on sexual differentiation of the male embryo. They have a definite masculinizing effect on the female embryo, however. Development of male structures is stimulated and development of some normal female

TABLE 1

Group No.	Substances and Doses (in Mg.)	Ratio And./Est.	Newborn		Serially Sect.	
			♂	♀	♂	♀
11.....	Test. prop.* 1.0 est. benz.† 15.0	1:15	8	3	2	2
10.....	Test. prop. 3.0 α estradiol 15.0	1:5	5	15	3	3
9.....	Test. prop. 7.0 α estradiol 17.5	1:2.5	6	6	3	3
2.....	Test. prop. 14.0 α estradiol 35.0		2	3	2	2
3.....	Test. prop. 14.0 est. benz. 28.0	1:2	4	8	3	3
5.....	Test. prop. 14.0 α estradiol 14.0	1:1	13	22	5	5
6.....	Test. prop. 14.0 α estradiol 7.0	2:1	22	14	5	5
7.....	Test. prop. 14.0 α estradiol 3.5	4:1	19	7	5	4
Totals	.....	.....	79	78	28	27

* Testosterone propionate. † Estradiol benzoate.

structures is inhibited. The Wolffian ducts, instead of degenerating as they do in the normal female embryo, persist and differentiate into well-developed epididymides, vasa deferentia, and seminal vesicles. The urogenital sinus develops into a typical male type of urethra with the various prostatic components (coagulating gland, dorsal and ventral prostrates). Since female differentiation of the urogenital sinus is prevented, no urogenital sinus portion of the vagina (caudal portion) is formed. The external genitalia are also typically male in type. Nipples are completely absent and never develop even post partum. Development of the Mullerian duct derivatives is not appreciably modified by androgens; thus the oviducts, uteri, and cranial portion of the vagina are present.

B. ESTROGENS

Estrogens given to the pregnant rat feminize the male embryo in that the male type of differentiation of sexual structures is inhibited and the female type is stimulated. Characteristic development of the Wolffian duct is prevented. There are very few or, more usually, no epididymal convolutions and the seminal vesicles are relatively minute. Inhibition of the Wolffian duct may be even more extensive, so that portions corresponding to the epididymis or vas may have degenerated and are thus absent at birth.

The Mullerian duct in the male embryo is stimulated to variable degrees by the estro-

gens. Instead of degenerating as in the normal male, parts of this duct, corresponding to the oviduct and uterus, may be present. The more caudal portions of the Mullerian duct are invariably stimulated to form a well-developed cranial (Mullerian) portion of the vagina. There is some differentiation of the urogenital sinus in the female direction, so that the upper part of the caudal portion of the vagina is also formed. Male differentiation of the urogenital sinus is prevented, so that no prostatic anlagen are formed.

The external genitalia of these newborn males are identical with those of newborn females. A complete set of well-developed nipples is also present—a condition which normally never occurs in the male and, even in the female, occurs only during post partum development.

Estrogens cause some paradoxical changes in the development of sexual structures in the female embryos. The Wolffian ducts (which normally degenerate in the female) may persist in estrogen-modified animals, either entirely or in part. These ducts, however, do not undergo epididymal differentiation, but minute outpouchings similar to the early anlagen of the seminal vesicles have been noted in a few females. In addition, development of the terminal part of the caudal (urogenital sinus) portion of the vagina is inhibited. The vagina of an estrogen-modified newborn female does not resemble that of an androgen-modified female, however, but is identical with that of an estrogen-modified male.

C. EFFECTS OF COMBINED ESTROGENS AND ANDROGENS ON EMBRYONIC DEVELOPMENT

When the two hormones are administered together to the pregnant rat, they display mutually antagonistic effects on the developing sexual structures of the embryo. The exact effect depends on the relative amounts of the two hormones administered. With certain dosage combinations one hormone will have no effect, the sexual structures of the embryo responding as if only the other hormone had been administered. With a higher dosage level of the first hormone, the effects of the second are inhibited or diminished. With a still higher dosage level of the first hormone, the effects of the second are completely antagonized or nullified.

Different sexual structures may vary somewhat in their sensitivity, i.e., as to exactly what proportions of estrogen and androgen are necessary to produce certain changes. For example, in some males, with a certain proportion of the two hormones the coagulating glands develop normally and thus give no evidence of being affected by the estrogen, while the ventral prostate is absent and thus gives evidence of full estrogen inhibition. The response of different animals of the same dosage group, however, has been fairly uniform, certainly as uniform as would be expected in a procedure of this type.

Those effects of one hormone which have been completely nullified by the other hormone are listed as follows:

1. Specific androgen effects in the female which are completely nullified by an adequate amount of estrogen

	Androgen	Androgen + estrogen
a)	Full development of epididymis	No development of epididymis
b)	Full development of seminal vesicles	Inhibited development of seminal vesicles
c)	Full development of coagulating glands	Coagulating glands absent
d)	Full development of dorsal prostate	Dorsal prostate absent
e)	Full development of ventral prostate	Ventral prostate absent
f)	Male type of external genitalia	Female type of external genitalia
g)	Urogenital sinus vagina absent	Upper part (only) of urogenital sinus vagina present

2. Specific estrogen effects in the female which are completely nullified by an adequate amount of androgen

	Estrogen	Estrogen+androgen
a)	Precocious development of nipples	No development of nipples
b)	Development of upper part (only) of urogenital sinus vagina	No development of any part of urogenital sinus yagina

3. Specific estrogen effects in the male which are completely nullified by an adequate amount of androgen

	Estrogen	Estrogen+androgen
a)	Inhibition of epididymal development	Full epididymal development
b)	Degeneration of portions of vasa	Complete development of vasa
c)	Seminal vesicles rudimentary	Full development of seminal vesicles
d)	Coagulating glands absent	Coagulating glands present and fully developed
e)	Dorsal prostate absent	Dorsal prostate present and fully developed
f)	Ventral prostate absent	Ventral prostate present and fully developed
g)	Formation of upper portion of urogenital sinus vagina	No formation of urogenital sinus vagina
h)	Portions of Mullerian ducts corresponding to oviducts and uteri present	These Mullerian remnants absent
i)	Female type of external genitalia	Male type of external genitalia
j)	Nipples well developed at birth	No nipples formed

It is obvious that antagonism between the two types of hormones cannot be demonstrated when one of the hormones has no effect on a certain structure or when both hormones produce similar effects on a structure. Androgens alone have no effect on certain Mullerian duct derivatives in the female (oviduct, uterus, and upper vagina); consequently, estrogens cannot be expected to demonstrate an antagonistic effect on these structures. Androgens invariably cause persistence of that part of the female Wolffian duct corresponding to the vas deferens. Estrogens, however, also cause partial or complete persistence of this portion of the Wolffian duct in some females. The two types of hormones, therefore, cannot be considered antagonistic in their effects on this structure.

In well-modified males, portions of the Mullerian duct may be developed by estrogens, but this finding has been inconsistent and has shown no correlation with the dosage of estrogen administered (3.0–100 mg. α estradiol, estradiol dipropionate, or stilbestrol). Because of this inconsistency in the stimulation of the Mullerian duct by estrogens, the absence of Mullerian remnants in males exposed to combined estrogen and androgen is not necessarily an expression of antagonism between the two hormones.

In the presentation to follow, the sexual structures to be considered are limited to those which have shown consistent and predictable responses to either of the two hormones when given alone. Since the object of this study is to determine whether the male and female differ in the response of their sexual structures to the antagonistic hormones, only structures which are capable of demonstrating an antagonism between the hormones will be considered. For this reason observations in both sexes on Mullerian duct derivatives and on the vas deferens will be omitted.

METHOD OF PRESENTATION

The findings in the male and female newborn are compared in Tables 2 and 3. The percentages of each sex showing typical androgen effects and the percentages of each

showing typical estrogen effects are recorded for each dosage group. In order to simplify the tables, the percentages of animals showing effects intermediate between these two

TABLE 2

EFFECTS OF COMBINED HORMONES ON WOLFFIAN DUCT DERIVATIVES EXTERNAL GENITALIA, AND NIPPLES

Dosage Ratio And./Est.	Epididymis				Seminal Vesicle				External Genitalia				Nipples			
	And. Effect		Est. Effect		And. Effect		Est. Effect		And. Effect		Est. Effect		And. Effect		Est. Effect	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
0:1	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**
1:15	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**
1:5	....	....	**100**	**100**	....	....	**67**	**50**	40	27	....	....	**60**	....	40	**100**
1:2.5	....	....	**67**	**57**	20	....	**60**	40	**75**	**78**	....	....	**88**	**78**	12	22
1:2	**72**	....	11	**77**	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....
1:1	**80**	....	7	**53**	40	**80**	10	....	**100**	**100**	....	....	**100**	**100**	....	....
2:1	**80**	23	7	40	**90**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....
4:1	**100**	**90**	....	13	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....
1:0	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....

And. = androgen; Est. = estrogen.
Figures represent percentage of findings in males or females of each dosage group.

TABLE 3

EFFECTS OF COMBINED HORMONES ON UROGENITAL SINUS DERIVATIVES

Dosage Ratio And./Est.	Coagulating Gland				Dorsal Prostate				Ventral Prostate				Urogenital Sinus Vagina			
	And. Effect		Est. Effect		And. Effect		Est. Effect		And. Effect		Est. Effect		And. Effect		Est. Effect	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
0:1	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**
1:15	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**
1:5	....	....	....	....	....	....	33	33	....	....	**100**	**100**	**100**	**100**	....	....
1:2.5	**60**	....	....	....	....	....	....	20	....	....	20	**80**	**100**	**100**	....	....
1:2	**100**	**100**	....	....	33	33	....	....	33	....	33	33	**100**	**100**	....	....
1:1	**80**	**60**	....	....	**60**	**60**	....	....	40	20	20	20	**100**	**100**	....	....
2:1	**100**	**100**	....	....	**80**	**80**	....	....	40	....	....	40	**100**	**100**	....	....
4:1	**100**	**100**	....	....	**80**	**100**	....	....	**60**	**100**	....	....	**100**	**100**	....	....
1:0	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....	**100**	**100**	....	....

And. = androgen; Est. = estrogen.
Figures represent percentages of findings in males or females of each dosage group.

extremes are not included. They may be calculated from the presented data if desired. In the tables, percentages of 50 or more are shown in bold type.

The findings in the epididymis were presented for each component (caput, corpus, and cauda) in a previous publication (Greene, Burrill, and Ivy, 1941). For the purpose of simplifying the presentation, the epididymis is considered as a whole in Table 2. The percentage values in each dosage group have been calculated from the averages of the three separate components.

In most structures the findings labeled "androgen effect" correspond to those in a normal male and those labeled "estrogen effect" correspond to those in a normal female. However, this is not completely true in the case of the epididymis. With the androgen effect this structure is identical with the normal male structure, but estrogen effect does not necessarily signify its absence as in a normal female. As previously mentioned, estrogens may cause either complete absence of the epididymis in the male or inhibition of its differentiation, in which case the epididymal duct may remain but have few or none of the convolutions characteristic of this structure. Accordingly, in the present series, males with intact epididymal ducts having few or no convolutions are included under estrogen effect. As also previously mentioned, estrogens in the female may cause persistence of a simple, nonconvoluted portion of the Wolffian duct in the epididymal region. Accordingly, females with this portion of the Wolffian duct present but not convoluted have been listed under "estrogen effect."

In some cases the derivation of the percentages may seem obscure. For example, in Table 2 under "seminal vesicle," the percentage of males showing a full estrogen effect in the 1:1 dosage group is given as 10 per cent. Since there were 5 males in this group, the conclusion is that one-half a male showed the full estrogen effect on the seminal vesicle. Since it has been demonstrated before (Burrill, Greene, and Ivy, 1940) that the two sides of bilateral structures may differ in their response to hormones, this conclusion is correct. One of the males in this group had the typical estrogen inhibition of seminal vesicle development on only one side.

COMPARISON OF MALE AND FEMALE NEWBORN

I. EPIDIDYMIS

Table 2 shows that there is a marked difference between the males and the females in the response of the epididymal portion of the Wolffian duct to the combined hormones. In the male, full epididymal development (androgen effect) was obtained in the majority of animals with 1 part (or more) of androgen to 2 parts of estrogen. In the female, however, a much larger proportion of androgen had to be given to obtain similar findings. Full epididymal development was not obtained in the majority of females until four times as much androgen as estrogen was given.

There is also a difference between the two sexes in the proportions of the two hormones required to obtain a full estrogen effect on the epididymis. This effect was not obtained in the majority of males until a proportion of 1 part androgen to 2.5 parts estrogen was given. In the females relatively less estrogen was required. The majority of females showed the full estrogen effect with a proportion of 1 part androgen to only 1 part estrogen.

There were also differences between the males and the females which are not apparent in the table. Thus, 16 of the 27 females had part or all of one or both epididymides missing. This was true of only one of the 28 males. This finding is probably not significant, since the response of this portion of the Wolffian duct to estrogens differs in the two sexes.

However, the fact that only 4 females showed full bilateral epididymal development in response to androgens, while 13 males showed this full response, clearly indicates a difference between the two sexes.

II. SEMINAL VESICLE

There is probably no significant difference between males and females in the response of the seminal vesicles (Table 2). With a proportion of 1 part androgen to 2 parts estrogen, full development of the seminal vesicles (androgen effect) was obtained in both sexes. With larger proportions of estrogen there was some variability in response, but the estrogen effect predominated in both males and females.

III. PROSTATES

It is difficult to interpret the observations on the three parts of the prostatic complex (Table 3). The differences between males and females, when present, are minor and do not seem significant except in the case of the ventral prostates.

TABLE 4

RESPONSE OF PROSTATIC COMPONENTS IN MALE AND FEMALE EMBRYOS

	No. Animals	Ventral Prostate		Dorsal Prostate		Coagulating Gland	
		And. Effect (Per Cent)	Est. Effect (Per Cent)	And. Effect (Per Cent)	Est. Effect (Per Cent)	And. Effect (Per Cent)	Est. Effect (Per Cent)
Male	26	30.7	23.1	46.2	3.8	76.9	0
Female	25	20.0	44.0	48.0	4.0	60.0	0

And. = androgen; Est. = estrogen.

Since the dosage was the same for the males and the females in each group (usually litter mates), it was thought that the sum of the responses for the males compared with the sum of the responses for the females might indicate the presence or absence of valid differences between the two sexes. The animals of the 1:15 group (1.0 mg. testosterone propionate and 15.0 mg. α estradiol) were omitted from this comparison as the androgen dosage used is per se insufficient to cause the development of all prostatic anlagen in the female embryo. The sums of the responses (expressed as percentages of the total of each sex) in the remaining groups are shown in Table 4.

A slightly higher percentage of males than females had well-developed ventral prostates, while a definitely higher percentage of females than males had none. The ventral prostates of the two sexes, therefore, seem to show a significantly different sensitivity in response to the two hormones.

There was no significant difference between the males and the females in the condition of the dorsal prostate. Practically the same percentage of males and females had full development of this component or complete absence of it.

The males showed a higher percentage of fully developed coagulating glands than did the females. However, since it is difficult to estimate from serial sections whether the coagulating gland is normal or is slightly inhibited, the tabulated 16.9 per cent greater incidence of fully developed coagulating glands in the males is probably not so significant as it might appear. On the other hand, the presence of definitely inhibited coagulating

glands cannot be confused with their complete absence. None of the males or females of the groups being considered completely lacked coagulating glands. Four in each sex were considered to be markedly inhibited. The only actual difference between the two sexes was in the number which were judged to have normal or slightly inhibited coagulating glands. It therefore seems probable that a significant difference between the male and the female in the response of the coagulating glands has not been demonstrated by our procedure.

IV. UROGENITAL SINUS VAGINA

There was no difference between the males and the females in the development of the urogenital sinus portion of the vagina (Table 3). In both sexes the androgen effect prevailed (complete absence) until a ratio of 1:15 was reached, when the usual estrogen effect (partial presence) was obtained.

V. EXTERNAL GENITALIA

The external genitalia of both males and females showed essentially the same response to the combined hormones (Table 2). With a ratio of 1 part androgen (or more) to 2.5 parts of estrogen, the majority of males and females had fully developed male type of genitalia. At a ratio of 1:5 some males and some females showed the same full androgen effect, although the majority showed mixed male and female characteristics. Usually they had a long ano-phallic distance, which is characteristic of the normal male, combined with the presence of a partial hypospadias, which is characteristic of the normal newborn female.

VI. NIPPLES

There is a slight but probably definite difference between the males and the females in the effects of the hormones on nipple formation (Table 2). In the majority of males 1 part of androgen was sufficient to prevent the usual nipple formation by 5 parts of estrogen, while in the females 1 part of androgen inhibited only 2.5 parts of estrogen in this effect.

VII. SUMMARY OF DIFFERENCES BETWEEN MALES AND FEMALES

There is a very distinct difference between male and female embryos in the response of the epididymal portion of the Wolffian duct to estrogens and androgens. In the male this structure is more sensitive to the effects of androgens, but in the female it is more sensitive to the effects of estrogens. Only two other structures show definite response differences in the two sexes. The ventral prostate in the female is more sensitive to estrogens and less sensitive to androgens than it is in the male. Formation of nipples in response to estrogens is more readily effected in females than in males, and, conversely, the androgens more readily inhibit this estrogenic effect in the males than in the females.

Our procedure has not demonstrated a significant difference between males and females in the effects of the two types of hormones on the formation of seminal vesicles, dorsal prostate, coagulating glands, urogenital sinus portion of the vagina, or the external genitalia. It is possible that, with a more sensitive procedure than the one we have used, sex differences in the responses of these structures might also become apparent.

DISCUSSION

The sex difference in the response of the Wolffian duct to the combined hormones is apparent only in its cranial derivative, the epididymis; while its caudal derivative—the

seminal vesicle—shows no sex difference in its response. These facts are evidence against an assumption that the Wolffian duct as a whole possesses an inherent sex specificity in hormone sensitivity. It seems unlikely that only the cranial portion of the duct would possess such a presumed inherent sex specificity in its response, i.e., a greater sensitivity to androgens in the male and a greater sensitivity to estrogens in the female. The following is an explanation as to why the epididymis in its response differs not only from the seminal vesicle but also from all the other sexual structures. This sexual structure which shows a marked sex difference in its response to the administered sex hormones develops in close proximity to the gonad, while those structures which show only a minor sex difference or none at all are more distant from the gonad. It therefore seems possible that some substance produced by the embryonic gonad has diffused through and affected adjacent tissues. In the female this substance apparently enhances the effect of the administered estrogen and minimizes the effect of the administered androgen. In the male the opposite is true, the substance enhances the androgen effect and minimizes the estrogen effect. While not generally known, there is excellent evidence from local-application studies (Zondek, 1935; Jadassohn, Uehlinger, and Zuescher, 1937; Greene and Wigodsky, 1938) that sex hormones are transportable to a certain extent by diffusion. It therefore seems reasonable that these hypothecated embryonic gonadal substances, which enhance the effects of the administered hormone characteristic for each sex, actually may be sex hormones. Whatever their nature, they apparently exert a greater influence on the epididymis than on other sexual structures. This, we believe, is because the epididymis is nearest to the gonad and is subjected to the greatest concentration of this substance, i.e., that which reaches it by diffusion *plus* that which is transported to it by the blood stream. Other structures, which are beyond the outer limit of the diffusion gradient, are reached only by the blood-borne substance. These structures are therefore subjected to a smaller concentration of the embryonic gonadal substance characteristic for each sex and consequently would manifest less sex difference in their response to administered hormones. It is possible that a procedure more sensitive than the one which we have employed might show, in all these structures, a definite effect of the blood-borne substance.

Moore (1941) has recently presented a "working hypothesis" which is in complete disagreement with the foregoing suggestion. He expresses the view that differentiation of the sexual ducts and accessories is not controlled by "gonad-secreted hormones" but rather is under the control of genetic factors, to which may be added "certain stimulating humoral substances [which] issue from the entire somatic complex of the organism." His reasons for rejecting any gonadal-hormone concept are based on his interpretation of the results obtained by himself and others from administrations of sex hormones to animals undergoing sexual differentiation. He concludes that "the postulated stimulation of homologous characters and inhibition of heterologous characters [by administered hormones] does not hold." His review of the experimental findings, however, is selective and, as a consequence, incomplete. For instance, he stresses the facts that in the guinea pig, rat, mouse, and opossum, although androgens stimulate the Wolffian duct of the female, they do not inhibit the Mullerian duct and that estrogens may cause persistence of the Wolffian duct in the female rat and opossum. However, he does not mention that, in both the rat and the mouse, estrogens inhibit the Wolffian duct of the male and that they also stimulate the Mullerian duct in the male and the female. Furthermore, the apparently sex-specific effects which estrogens and androgens have displayed on the external genitalia, nipples, and most urogenital sinus structures should not have been disregarded.

Androgens, for example, cause differentiation of the prostate in the female rat and mouse, while estrogens prevent formation of the prostate in the male. Moore observed these effects in the opossum but chose to interpret the incomplete development or complete absence of the prostate in the male opossum under the influence of estrogens as not a "true inhibition" but only an inhibition of the "type of differentiation." We concur with this last interpretation but consider the effects of administered hormones on the type of embryonic differentiation to be the main issue involved in any evaluation of the factors controlling sexual differentiation.

In the female rat, androgens cause retention of the Wolffian duct and stimulate it to develop in a typical male fashion, while, in the male, estrogens stimulate the Mullerian duct and inhibit the typical male type of differentiation of the Wolffian duct, often causing its actual degeneration and partial disappearance. These effects, we contend, can be regarded as evidence for homologous stimulation and heterologous inhibition. It is true that not all sexual structures demonstrate a consistent specificity in response to administered sex hormones. Moore has cited all such instances. However, the majority of sexual structures do show a sex-specific type of response, and in formulating theories such responses should be considered.

Moore has also suggested the possibility that departures from normal development in the offspring of pregnant rats treated with sex hormones during pregnancy might simply represent abnormal development as a direct result of nonspecific placental damage. Inasmuch as the changes noted in these offspring have generally been confined to the reproductive system and have differed qualitatively according to the specific hormone administered, we feel that these effects can hardly be considered as nonspecific. The existence of such a nonspecific damaging effect seems even less likely since entirely normal adult male rats have been derived from mothers receiving large amounts of androgens during pregnancy, these males having been intra-uterine coresidents of markedly masculinized females which also grew to healthy adulthood. Furthermore, such a nonspecific toxic mechanism would hardly explain the fact that, by combined administration of appropriate amounts of estrogens and androgens, most of the effects of one can be prevented by the other. It is difficult to conceive of two nonspecific toxic effects which are mutually and specifically antagonistic.

In a recent review Witschi (1941) expressed the following opinion concerning the effects of estrogens on the rat embryo as reported by us: "It appears that specific stimulation effects are practically absent and that the observed abnormalities may be considered as unspecific inhibitions leading to persistence of embryonic conditions." This point of view, we feel, is not entirely justified. In male rats treated prenatally with estrogens parts of the Mullerian duct are retained, and in the females the Wolffian duct is retained either partially or completely. These effects of the estrogens, it is true, might be considered as representing "persistent embryonic conditions." However, the actual disappearance of parts of the Wolffian duct in the male under the influence of estrogens cannot be considered a persistence of embryonic conditions. It is a definite departure from embryonic conditions and represents the result of an embryonic process not found in the male but characteristic of female development. Similarly, the formation of a partial lower vagina in the male treated with estrogens is a process not found in male development, but rather a process typical of female development. While simple retention of the Wolffian duct in the female may be considered an expression of arrested development, the formation of seminal vesicles in females cannot be so considered. No anlagen for these

structures are ever formed in the normal embryonic development of the female. Moore's review to the contrary, we have stated in two previous papers (1939*a*, 1940) that small, but definite, seminal vesicles were found in some of the estrogen-treated females. Although their development in response to administered estrogens is inexplicable, their presence in the female certainly cannot be interpreted as a manifestation of developmental arrest.

The primary purpose of experimental work on sexual differentiation is to determine what factors are responsible for normal differentiation and how they operate. There is abundant evidence, in a variety of animals, that sex hormones can influence the course of sexual differentiation. The chief difficulties in using this evidence to formulate a theory as to the actual role of the sex hormones in normal development are the occasional conflicting and sometimes paradoxical actions of these administered substances in different species or even in the same animal. Actually, the experimental work furnishes evidence only for the responses of the embryonic sexual structures to the administered sex hormones which are similar to, or presumably identical with, the hormones produced in the adult gonads. Strictly speaking, one cannot interpret the effects of such administered hormones in terms of the possible effects of endogenous hormones of the embryo without assuming that the two types of hormones are identical in nature and effects. This assumption may not be valid. The presumed embryonic hormones may differ chemically from the adult hormones and may differ also in some of their effects. If such a qualitative difference between embryonic and administered hormones exists, it would serve to explain certain differences between the naturally occurring freemartin and all experimentally produced mammalian intersexes. For example, in the freemartin, where the effective agent is presumably the endogenous embryonic hormone of the male twin, the Mullerian duct of the female is strongly inhibited. Such an effect has never been elicited by any of the administered "male" hormones.

Such a qualitative difference may also explain why the experimental evidence does not support in every detail a conclusion that normal embryonic sexual differentiation is under the direct and exclusive control of sex-specific hormones produced by the embryonic gonads. Absolute negation of such a hypothesis does not seem warranted, however, in view of the large proportion of the evidence which tends to support it.

In the course of studying the mutual antagonism displayed by sex hormones in their effects on differentiating sexual structures of the rat, we have encountered a situation (the epididymis) which we believe reflects an active participation on the part of the embryonic gonads. We do not wish to be interpreted as stating that this particular finding proves the gonadal hormone theory of embryonic sexual differentiation. Nevertheless, we feel that it may be considered an additional factor in the support of such a theory.

SUMMARY

Estrogens and androgens, when administered simultaneously, are mutually antagonistic in their effects on developing sexual structures of the rat embryo. The responses of most sexual structures to the combined hormones are identical in the two sexes, i.e., similar effects are obtained in both the male and the female with approximately the same dosage ratios of the two substances. The epididymal portion of the Wolffian duct, however, shows a marked response difference in the two sexes. In the male this structure is more sensitive to the effects of androgens, while in the female it is more sensitive to the

effects of estrogens. To obtain similar effects on this structure in the two sexes a larger proportion of androgen is required in the female than in the male and a larger proportion of estrogen is required in the male than in the female. The ventral prostate and the nipples also exhibit a sex difference in response to the combined hormones. In these structures, however, there is less difference between the sexes than in the case of the epididymis. An interpretation of these findings is given, involving an active participation on the part of the embryonic gonads.

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DIFFERENTIAL DYE REDUCTION IN OVARIES OF DROSOPHILA HYDEI

(Twenty-one figures)

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IT HAS long been known that insect ovaries generally consist of follicular tubules, each containing a single series of stages of oögenesis with or without accessory or nurse cells. At one end of the tubule—the apical end—are the primitive oögonia, and successive stages of oögenesis follow in order along the tubule. Polar and ventrodorsal patterns are usually evident in the form of the egg; the polar axis coincides with the longitudinal axis of the tubule, the end of the elongated egg toward the apical end of the tubule becoming the apical or "animal" pole of the egg. The question whether or how the ventrodorsality of the egg is related to its ovarian environment seems to have received little attention. In view of the fact that a definite relation between egg polarity and ovarian environment has been found in many animals, the earlier widely held belief that axiate pattern is inherent in a species protoplasm has given way rather generally to the view that the egg acquires its axiate pattern during its ovarian history and in relation to factors in its ovarian environment, but evidence of relation between ventrodorsality or dorsiventrality and ovarian factors is, in general, less definite than that concerning polarity or is lacking entirely.

The question whether there is a physiological pattern in the insect ovary to which the axiate pattern of the egg is definitely related is of interest as bearing on the problem of egg organization, although presence of such a pattern does not demonstrate its adequacy or effectiveness in determining egg pattern. But, if coincidence in orientation of ovarian and egg pattern is uniform and constant and similar physiological characteristics appear in both, it becomes at least highly probable that egg pattern represents primarily a part of the ovarian pattern.

Differential reduction of "vital" dyes has been found useful as a means of demonstrating certain characteristics of physiological axiate patterns in embryonic stages of echinoderms, in the chick embryo, in ciliate Protozoa, in postembryonic stages of various animals, and in reconstitution in *Corymorpha* and other forms.[1] The morphological pattern of the insect ovary, with the various stages of oöcyte development from primitive germ cells to full-grown egg, suggested that it might be favorable material for a study of differential dye reduction in relation to axiate egg pattern.

I am deeply indebted to Dr. G. W. Beadle and Dr. E. L. Tatum of the School of Biological Sciences of Stanford University for their kindness in providing a stock of *Drosophila hydei*, culture bottles and food, a supply and formula of Ringer solution found satisfactory for insect tissues, sodium hydrosulphite used in certain experiments, and for various suggestions concerning handling of the stocks of living insects.

[1] Child, 1934*a*, protozoa, hydra, flatworms, oligochetes; 1934*b*, *Paramecium* and other ciliates; 1936*a*, *b*, developmental stages of echinoderms; Child and Rulon, 1936, oligochetes; Child and Watanabe, 1935, *Corymorpha;* Rulon, 1935, chick embryo; Watanabe, 1935, *Corymorpha*, reconstitution and dominance.

MATERIAL AND METHODS

The only insect species used thus far is *D. hydei*, selected in preference to other species of *Drosophila* because of its larger size. Since only very early follicles are present in ovaries of newly emerged imagos, it has not been found necessary to use ovaries of pupal stages. For removal of ovaries, flies are allowed to enter a small bag of fine netting placed over the mouth of the stock bottle. There they are killed or immobilized by slight pressure. Anethesia has been avoided because of possible effect on physiological condition of the ovaries. The flies can be immobilized by refrigerator temperature and killed by pressure, but the netting bag is more satisfactory because the desired number can be obtained in a few seconds at any time.

Even the very small ovaries of newly emerged flies, though only 0.2–0.3 mm. in length, are readily distinguished from other tissues under low magnification by their opaque white appearance and are easily isolated with needles. All dissections were made in the insect Ringer solution recommended by Dr. Beadle, NaCl, 6.075 gm.; KCl, 0.283 gm.; $CaCl_2$, 0.170 gm.; H_2O, 1 liter, and ovaries were not removed from it at any time. Since they could be isolated very rapidly, often a pair in a few seconds, a number, 8, 10, in some cases 15 or 20, of the small earlier stages were usually prepared and stained as a single lot. These small early ovaries were either stained and reduced intact or teased with fine needles to separate, single follicle strings and follicles. The large ovaries of later stages 1.5 mm. or more in length, with full-grown eggs in their basal regions and early follicles apically, were usually more or less teased to separate eggs and follicle strings so that their reduction could be followed individually; or in some cases the early follicles of apical regions were separated from the large mass of eggs in the basal region by a transverse section. More than a thousand ovaries were used in the experiments.

For staining, methylene blue and Janus green (diazine green) in insect Ringer were used. In dye-reduction experiments use of a wide range of dye concentrations is necessary in order to make certain that differential depth of staining or differential injury by the dye are not concerned in the reduction pattern observed. Moreover, certain interesting results appear with very low dye concentrations. Concentrations of methylene blue ranged from 1/20,000 to 1/200,000 and of Janus green from 1/30,000 to 1/600,000 and staining periods from 5 minutes to several hours or, in very low concentrations, a day or two. With both dyes injury may result from overstaining, and reduction may be greatly retarded or not occur at all in follicles thus injured. Moreover, regions which normally reduce the dye most rapidly are most susceptible to injury and when injured may reduce less rapidly than less susceptible regions, so that the reduction differential is reversed in direction along the ovarian axis.

Dye reduction has been brought about by gradual decrease of oxygen in the solution containing the ovaries, through oxygen consumption of the ovaries, or with other tissues added to bring about more rapid decrease. Gradual decrease rather than sudden removal of oxygen is desirable for demonstration of reduction differentials. With immediate establishment of anaerobic conditions, reduction may occur so rapidly in all parts that reduction differentials clearly evident with gradual oxygen decrease do not appear at all. For gradual oxygen decrease the following procedures were employed: (*a*) ovaries in varying number, with or without other tissue, were sealed by a cover glass in a small cell made by a ring of vaseline on a slide; (*b*) ovaries were placed on vaseline, most of the fluid was removed, and they were pressed into the vaseline by a cover glass; (*c*) a

group was placed on a slide with little fluid and covered with mineral oil; (*d*) in some cases they were merely sealed in solution under a cover glass by running melted vaseline around it. The small cell with added tissue has been found most satisfactory because, even with the very small ovaries, dye reduction can be made to begin within a few minutes after sealing, while the ovaries are still in good condition, but is nevertheless gradual. With other methods oxygen decrease is less rapid and, as will appear below, the reduction differential may be altered.

Early and advanced follicles and full-grown eggs are not stained satisfactorily for reduction with the same dye concentration and staining period. A concentration and period that stain the early follicles do not stain the late follicles and full-grown eggs sufficiently; and a concentration and period adequate for late follicles and eggs usually injure or kill the early follicles so that they reduce very slowly or not at all. If ovaries are returned to Ringer after staining, there may be more or less loss of dye with a basipetal differential, consequently it has been found desirable, particularly with methylene blue, to bring about reduction in the dye solution used for staining, so that loss of color is certainly due to reduction rather than to loss of dye. However, with methylene blue it can readily be determined whether loss of color in the low oxygen preparation is due to reduction or to loss of dye. Reduced dye regains its color on exposure of the solution to air, but after loss of dye there is, of course, no change or, after partial loss, less color than after the original staining. With Janus green decoloration from loss of dye and the red color of partially reduced dye are directly distinguishable. With very low dye concentrations adsorption of dye on the vaseline or glass may decrease concentration sufficiently so that gradual loss of dye from the ovaries takes place. Moreover, some differential decoloration occurs gradually in both dyes in very low dye concentrations with large volume of solution open to air and with frequent renewal to prevent decrease of concentration by adsorption. Cases of this sort and their significance are discussed in a later section. For most purposes it has been found desirable to seal the ovaries in the dye solution used for staining within 1 or 2 minutes, or at most 4 or .5 minutes, depending on dye concentration, after bringing them into the dye. With this procedure, staining and oxygen decrease go on at the same time, and there is less danger of injury from overstaining.

Minute quantities of $Na_2H_2S_2O_4$ have been found useful as an aid to rapid dye reduction by decreasing oxygen content of the solution. Because of the rapid decomposition of this agent in presence of oxygen no attempt has been made to use it in definite concentrations but extremely minute quantities, a few granules, added to 1 or more ml. of dye solution containing stained ovaries and open to air, bring about reduction, according to concentration, practically instantaneously, in a few seconds or in 1 to several minutes, with the same differential as with oxygen decrease by the tissues alone. With still lower concentrations only partial reduction may occur before the agent is completely oxidized.

The younger ovaries usually float because of air in the abundant tracheal supply, but but as they increase in size they become heavier and sink in spite of the tracheal air.

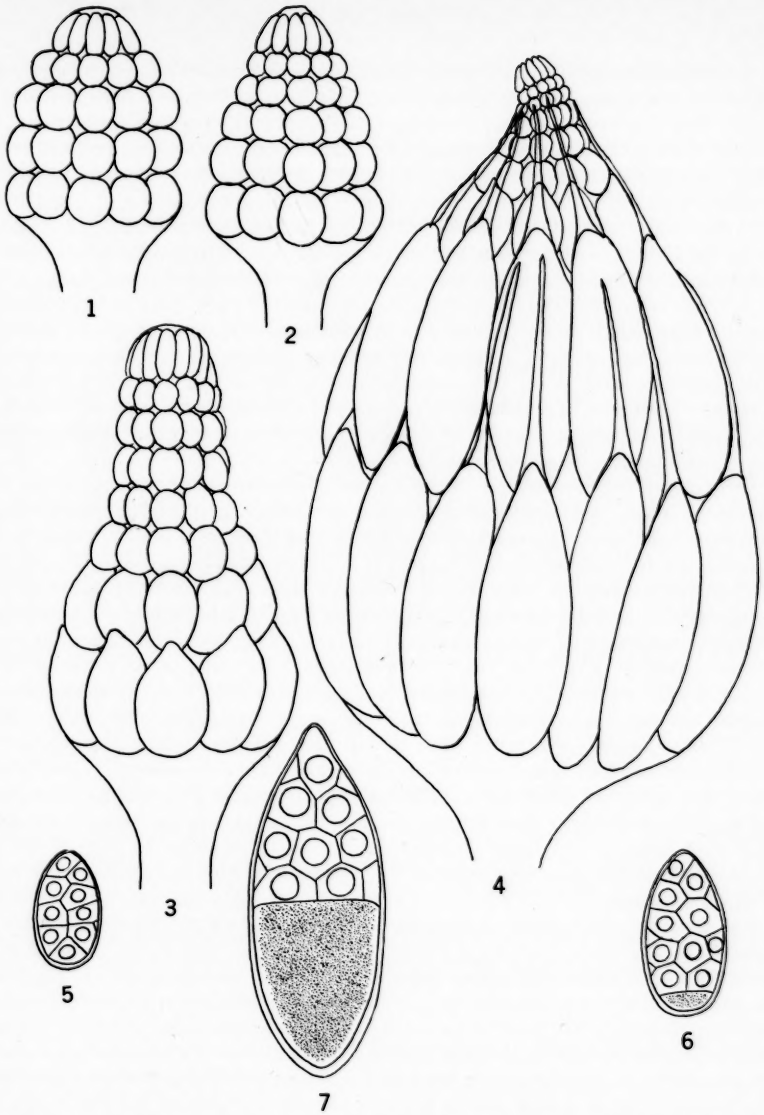
In dye solutions both younger ovaries and isolated ovarian eggs tend to stick to glass and other surfaces and are not infrequently lost in pipettes. After they have been sealed in cells, their positions usually cannot be altered by gravity in order to equalize oxygen content of the fluid. When two ovaries lie close together or when one lies close to other tissue, reduction is more rapid in the region adjoining the other ovary or tissue than elsewhere, but it is almost always possible to distinguish these group or mass effects

from the true reduction picture. When other tissues are added, their positions can usually be changed by gravity so that they serve to stir the solution and prevent local differences in oxygen content.

Figures are diagrammatic but are drawn from ocular micrometer measurements of whole ovaries, follicles, and eggs. Relative sizes of follicles and eggs are as measured in the living ovaries. Tracheal branches and their distribution within the ovaries are not shown. In Figures 1–3 and 5–7, of young ovaries and single follicles, and Figures 15–21, outlines of young ovaries and follicles with course of dye reduction indicated, the magnification is approximately 150, in Figures 4 and 8–14, advanced stages of ovarian development and full-grown eggs, it is half that (×75). In other words, the increase in size from earlier to later stages is twice as great as Figures 4 and 8–14 show in comparison with other figures. Since the course of dye reduction can be adequately shown only in series of figures in color, it is diagrammatically indicated by arrows showing the general directions of progress and by successive broken lines indicating stages. Boundaries between reduced and unreduced regions are, of course, not sharp; reduction in progress is always a gradient, except perhaps in the nurse cells of older follicles where it seems often to occur at about the same time in all those of a single follicle.

DEVELOPMENTAL STAGES OF THE OVARIES

The two ovaries lie side by side in the abdomen with apical ends anteriorly but with longitudinal axes diverging somewhat. They consist of follicle strings, each composed of a single series of follicles, with progress of development from follicle to follicle basipetally in the string. The follicles originate by successive constrictions near the basal end of a primary apical region of each ovarian tubule containing the primitive germ cells and growing in length more or less continuously. The group of follicle strings constituting an ovary is inclosed in a delicate, somewhat contractile, membrane, not indicated in the following figures. Each ovary connects basally with an oviduct, the two uniting a short distance posterior to the ovaries. Figures 1 and 2 show follicle outlines in ovaries of recently emerged flies. There is usually more or less regularly progressive increase in size of follicles basipetally, but relative rates of increase and stages of rapid increase at certain levels differ in ovaries of different individuals, though they are closely similar in the two ovaries of a pair. They determine the general form of the ovary. In Figure 1, for example, increase in follicle size is almost wholly in the two most apical follicles separated from the elongated apical region, which does not enlarge as a whole but gives rise to one follicle after another from its basal region by constriction. In Figure 2 increase in follicle size is more uniformly progressive, and the ovary as a whole is consequently more nearly conical in outline than in Figure 1. Figure 3 shows an ovarian form frequent in somewhat more advanced stages. Here there is little increase in size in the earlier follicles, but in the basal region of the ovary a relatively great increase in size appears in the last three follicles with corresponding increase in diameter of the ovary. Ovaries of conical form with uniformly progressive increase in follicle size also occur in these more advanced stages, and between the two extremes of form all gradations appear. The very extensive tracheal development in the young ovary in relation to its size suggests an intense metabolism. A varying number of tracheal branches enter the ovary near the middle of its length or at two levels and ramify extensively between the follicle strings. The tracheae are much more conspicuous in the young ovaries than in later stages. Whether the number of follicle strings in an ovary is always the same has not been determined.



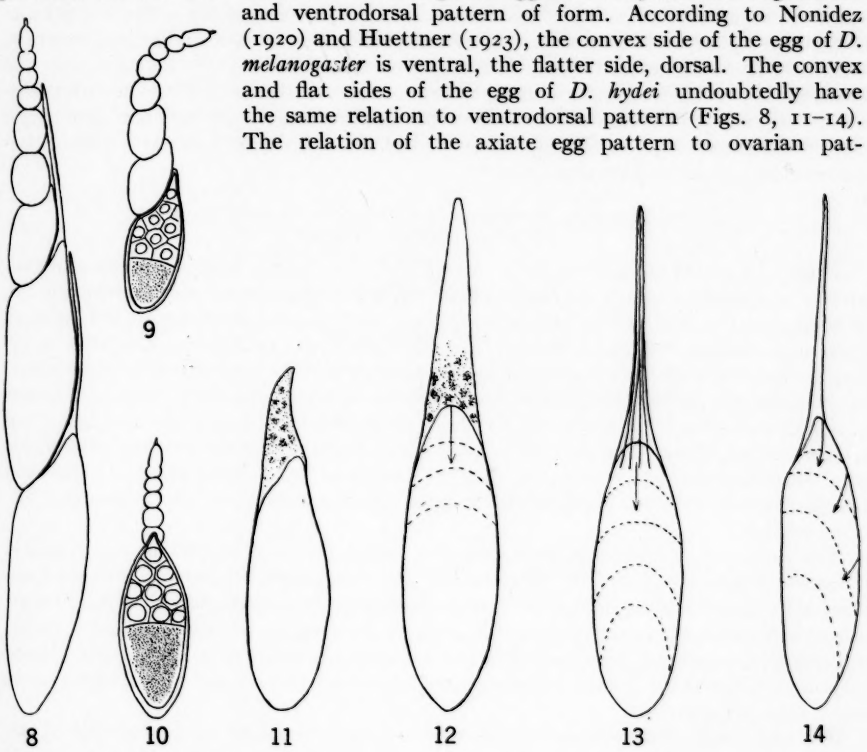
FIGS. 1–7.—Developmental stages of ovaries and single follicles of *Drosophila hydei*. Figs. 1 and 2, ovaries of recently emerged flies. Fig. 3, somewhat later stage of ovarian development. Fig. 4, advanced ovary with two series of full-grown eggs, drawn to a scale (× 75), half that of ovaries of Figs. 1–3 and 5–7 × 150); the prolongations of the chorion, which extend apically, separate longitudinally into four appendages. Fig. 5, an isolated follicle about 0.12 mm. in length, basal cell not yet distinguishable as oöcyte from nurse cells (pre-oöcyte stage). Fig. 6, isolated follicle 0.17–0.18 mm. in length, basal oöcyte becoming finely granular (early-oöcyte stage). Fig. 7, about 0.36 mm. in length with oöcyte occupying half its length (half-oöcyte stage).

A late ovarian stage with two series of full-grown or almost full-grown eggs is shown in Figure 4. The great increase in size during ovarian development is evident from the fact that Figure 4, as noted above, is drawn to a scale half that of Figures 1–3. In Figure 4 and in all other late ovarian stages seen thus far the apical regions are like young ovaries, the earliest stages of follicle development being still present, even though there are one or two series of full-grown eggs basally. In the more advanced ovarian stages there is also much variation in size of the follicles immediately preceding the full-grown eggs. In Figure 4 these are still relatively early follicles. In some late ovaries they may be much further advanced, for example, as in Figure 7, or there may be stages in disappearance of the nurse cells, as in Figures 11 and 12, between the younger follicles and full-grown eggs. Little or no difference in developmental stage at the same level of different follicle strings of a single ovary has been seen; consequently the successive stages usually form successive zones from apex to base of the ovary. Occasionally, however, ovaries have been seen in which full-grown eggs of a second zone, or some of them, appear to be pushing past or between eggs of the basal zone, so that overlapping or irregularities in the zonal arrangement result.

In early living follicles neither nuclei nor cell boundaries are clearly visible, but in follicles 0.1–0.2 mm. in length they can usually be seen (Figs. 5 and 6). From examination of later follicles it appears highly probable that the basal cell in Figure 5 is the oöcyte, though at this stage it does not differ in appearance from other cells of the follicle, all of which are nurse cells. For convenience these earlier follicle stages are called "pre-oöcyte follicles." At the stage of Figure 6 the basal cell is becoming finely granular and opaque, presumably from deposition of yolk, and the nucleus is no longer distinguishable with certainty. This is an "early-oöcyte stage." Figure 7 shows a later follicle in which the oöcyte occupies the basal half ("half-oöcyte follicle"). At this stage nuclei of the nurse cells are clearly visible, but cell boundaries are usually less distinct than in earlier follicles. Figures 5–7 do not show all the nurse cells or nuclei but only those near the upper side of the follicle as it lies. What the total number of nurse cells is and whether it varies have not been determined. The wall of the follicle increases in thickness basipetally, and its basal half, more or less, becomes thicker during earlier stages of growth of the oöcyte (Figs. 5–7). Tissue connections between successive follicles of the same string are slight in the stages of Figures 5–7; the follicles separate readily with no evidence of torn tissues.

Sudden transition from early to advanced stages is very clearly shown in isolated follicle strings. In Figure 8, from an advanced ovary, an egg, almost or quite full grown, follows a follicle of about the stage shown in Figure 5. In Figure 9 the transition is somewhat less abrupt; the most basal follicle shown, about the stage shown in Figure 7, a half-oöcyte follicle, is followed by a full-grown egg. Occasionally ovaries are found in which a sudden transition in stage appears much earlier. In the case of Figure 10 there is almost no increase in size in the first 6 follicles, but the seventh is a half-oöcyte follicle. Probably these differences in stage of successive follicles in later ovarian development are, like the differences in relative rates of increase in follicle size in early stages, results of differences and changes in nutritive conditions. In the stocks of flies kept for reduction experiments, change to fresh food supply was not always made immediately after emergence. The differences could perhaps be controlled experimentally by time of change to fresh food supply, perhaps also by controlled change of temperature at different stages.

Eggs with remains of nurse cells and developing apicodorsal chorionic appendages are outlined in Figures 11 and 12 and full-grown eggs in Figure 13 (dorsal view) and Figure 14 (lateral view). The chorionic appendages extend apically from a level near the apical pole somewhat on the dorsal side. The full-grown egg has a very definite longitudinal and ventrodorsal pattern of form. According to Nonidez (1920) and Huettner (1923), the convex side of the egg of *D. melanogaster* is ventral, the flatter side, dorsal. The convex and flat sides of the egg of *D. hydei* undoubtedly have the same relation to ventrodorsal pattern (Figs. 8, 11–14). The relation of the axiate egg pattern to ovarian pat-



FIGS. 8–10.—Isolated follicle strings, drawn to a scale half that of Figs. 1–3 and 5–7. Fig. 8, from an advanced ovary, showing relative sizes of follicles and full-grown eggs. Fig. 9, a string with most basal follicle shown almost half-oöcyte stage. Fig. 10, an extreme example of sudden change in follicle size from very early stage to half-oöcyte.

FIGS. 11–14.—Late stages of oögenesis and full-grown eggs. Figs. 11 and 12, late oöcyte stages with remains of nurse cells. Figs. 13 and 14, full-grown eggs. Arrows and broken lines in Figs. 12–14 indicate general course of dye reduction.

tern is highly suggestive as regards the question of its origin. Morphologically the relation is directly evident. As already noted, the apical pole of the ovary is directed anteriorly in the body and that region of the oöcyte which becomes the apical or animal pole is always toward the apical pole of the ovary. The ventral side of the eggs which lie at the surface of the ovary is always outward, and this seems to be the case with all eggs. In the isolated follicle strings of Figures 8 and 9 the outer side is on the right (see

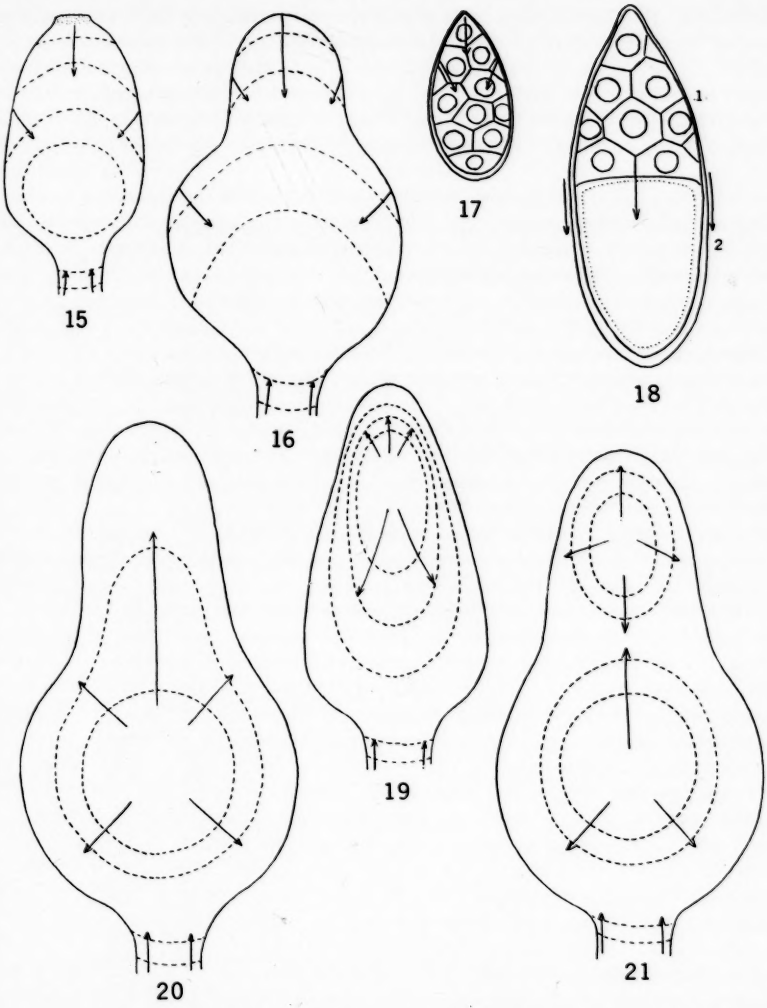
also Fig. 4). The 5 or 6 youngest follicles in the apical region of a follicle string are almost or quite spherical with no evidence of ventrodorsality. Usually, however, the sixth or seventh follicle begins to extend somewhat over the outer side of the follicle next apical to it. In later follicles this ventrodorsality becomes more distinct, and still later the chorionic appendages develop in association with it. This elongation of the ventral side of the developing follicle is shown in Figures 8 and 9. The oblique and slightly concave outline of the apicodorsal region of the full-grown egg in side view, together with the chorionic appendages, represents the final stage of this ventrodorsal difference in development (Fig. 14). In Figure 8 its earliest visible and final stages are indicated in a single string. The final shape of the egg, with ventral side convex and dorsal flattened, becomes evident only in advanced stages.

DIFFERENTIAL DYE REDUCTION IN INTACT OVARIES AND ISOLATED FOLLICLE STRINGS

It may be stated at once that dye reduction in low oxygen begins apically and progresses basipetally if young ovaries or isolated follicle strings are not overstained and are in good condition and that at a given level of an ovary there is also progress of reduction from outer surface inward. However, the early follicles are extremely susceptible to injury by the dye, with resulting retardation or absence of reduction. Reversal of direction of the reduction gradient may result from differential injury. In these cases retardation of reduction is greatest apically and less susceptible follicles 6, 8, or even more below the tip reduce first. From these reduction progresses acropetally, or the more apical follicles may not reduce presumably because they are dead or so injured that ability to reduce is completely lost. Other conditions affecting the course of reduction are discussed in another section.

The course of reduction in young ovaries under properly controlled conditions is indicated in Figures 15 and 16. The extreme apical regions of the youngest ovaries, indicated by slight shading in Figure 15, often stain slightly more rapidly and deeply than other parts and may begin to reduce slightly later than regions immediately below them; but this difference is not always evident and has not been observed in later stages. These extreme apical regions appear to be regions from which the ovarian tubules arise or to which they are attached.

How soon reduction becomes evident after sealing depends on oxygen content of the solution, volume of solution in relation to ovarian volume, rate of respiration of the ovaries, and rate of decrease of oxygen, which, of course, depends on the other factors. For example, 6–10 ovaries in stages of Figures 1–3, removed in insect Ringer and brought within a few minutes into Ringer methylene blue 1/30,000, left exposed to air for 5–10 minutes with frequent agitation to insure uniform staining, then sealed without air bubbles in a vaseline cell 3–4 mm. in diameter and only slightly deeper than the transverse diameters of the ovaries, usually show beginning of reduction in 15–30 minutes after sealing. In 1–1½ hours reduction is advanced, only basal internal regions showing color, or may be complete. On readmission of air, color returns at once, and with a second sealing reduction and reoxidation may be repeated. If other tissue is added for more rapid decrease of oxygen, reduction may begin 5–10 minutes after sealing and be complete in 15–30 minutes. These rates of reduction are for preparations at room temperature, 20°–22° C. At higher temperatures reduction becomes evident earlier and proceeds more rapidly, and at lower temperatures it is, of course, less rapid.



FIGS. 15–21.—Outlines of earlier ovarian stages and of follicles with arrows and broken lines and in Figure 18 numerals indicating directions and stages in progress of dye reduction under different conditions. Figs. 15 and 16, intact ovaries, not overstained, oxygen decrease rapid; dye reduction progresses basipetally and from outer surface inward in ovaries and acropetally a short distance from severed end of oviduct. Fig. 17, early-oöcyte follicle with basipetal progress of dye reduction. Fig. 18, half-oöcyte follicle, progress of reduction basipetal in follicle wall and in oöcyte; numerals 1 and 2 indicate that nurse cells reduce earlier than oöcyte; external boundary of yolk in oöcyte indicated by dotted line. Figs. 19–21, intact ovaries showing variations in course of dye reduction with slow oxygen decrease in dye solution.

The individual follicle strings of these young ovaries are easily separated more or less completely by needles. Except when the more susceptible apical follicles of the strings are injured by overstaining or undergo change in condition in consequence of isolation, reduction progresses basipetally, as in intact ovaries. No distinct gradient has been observed within individual follicles of early stages (usually the first 6, 7, or 8 follicles of a string), but the basipetal progress of reduction in the string as a whole is perfectly clear.

The reduction differential is very distinct; apical regions or the apical halves of ovaries or strings usually become completely colorless, while the basal regions are still deeply colored, and show only superficial or no appreciable reduction. In ovaries with more or less uniform progress of follicle development, as in Figure 2, the basipetal progress of reduction is usually also uniform. In those with slender apical and large basal region, as in Figure 3, reduction of the slender region, consisting of the youngest follicles, is usually relatively rapid as compared with that of the basal region.

Janus green is more toxic than methylene blue, and lower concentrations and shorter staining periods than with methylene blue have been found desirable. To avoid injury by the dye it is also desirable to bring about reduction soon after staining. The deeper the stain and the longer the period before reduction, the more frequent is retardation of reduction in the more susceptible regions and reversal in direction of progress or absence of reduction in the youngest, most susceptible follicles.

With concentrations of Janus green of 1/100,000 or lower, young ovaries, sealed at room temperature in small vaseline cells in the dye solution within 5–10 minutes after being brought into the dye and in sufficient numbers or with enough other tissue added so that reduction begins in 15–30 minutes, usually reduce basipetally and from the outer surface inward, as with methylene blue. The earliest stages of reduction of Janus green, indicated only by change from the bluish green of the oxidized dye to a slightly purple tint, are often best seen in a partially darkened field rather than with full illumination, apparently because the earliest change of color is at the surface of the ovary and is more clearly visible by reflected light in the darkened field but may be obscured in transmitted light in the fully illuminated field by the blue-green of deeper parts. With further progress of reduction the brilliant red color of the partially reduced dye and its basipetal progress are very distinct and striking. With continued oxygen decrease, further reduction from red to colorless appears apically and progresses basipetally and from the surface inward. The completeness of this second color change, associated with complete dye reduction, differs in different ovaries. In the youngest ovaries it may progress over the whole length; in those more advanced it may be limited to the more apical levels or may appear only as a greater or less fading of the red color apically with a basipetal gradient of increasing intensity of red. If the preparation is opened and exposed to air or fresh Ringer is added, the red color may return more or less completely with slight acropetal gradient, except under conditions described below where there is loss of dye. More or less return from colorless to red may also occur gradually in the sealed preparation. This is apparently associated with deterioration in condition of the ovaries and probably indicates approaching death; after it takes place no further change in color can be induced by opening or resealing after opening, though the apparently dead ovaries may gradually lose dye in Ringer.

It has not been possible to obtain basipetal reduction through the whole length in large advanced ovaries like that in Figure 4, because apical regions are usually injured

and their reduction is retarded or they do not reduce at all with dye concentrations and staining periods adequate for the full-grown eggs in the basal region. With proper staining and rapid oxygen decrease, reduction progresses basipetally down to the late follicles or full-grown eggs, but under such conditions the eggs are only slightly stained.

Reduction in single isolated follicle strings is similar to that in the strings of intact ovaries, but decrease in ability to reduce apparently occurs earlier in the isolated strings than in intact ovaries.

DYE REDUCTION IN SINGLE FOLLICLES

As noted above, no uniformly distinct differential has been observed in single, very early follicles, the first 6–8 of a string, probably in consequence of their small size. Reduction progresses basipetally from follicle to follicle, and in some of the young follicles a slight differential is visible. However, in follicles 0.1–0.2 mm. in length, before the oöcyte is visibly distinguishable, basipetal progress of reduction is characteristic; but this differential may be obliterated or reversed by overstaining. As follicle development and increase in size progress, the follicle wall shows a distinct basipetal reduction gradient, and the nurse cells reduce earlier than the oöcyte. In early-oöcyte follicles, like those in Figures 6 and 17, reduction usually progresses basipetally, as indicated by arrows and broken lines in Figure 17. In half-oöcyte follicles, like those in Figures 7 and 18, nurse cells reduce earlier than the oöcyte, usually with little difference among themselves, though sometimes slightly earlier reduction has been observed in the more apical cells. A slight basipetal reduction differential is characteristic of the oöcyte of these stages, as indicated in Figure 18. Here also reduction of the nurse cells may be retarded by overstaining, so that reduction in the oöcyte occurs or begins earlier than in the nurse cells. In later stages like Figures 11 and 12, the remains of the nurse cells still reduce earlier than the egg cell.

THE EGG

In the full-grown egg without visible injury dye reduction usually progresses posteriorly from the anterior end, that is, the end toward the apical end of the ovary; but the gradient is slight and may be altered by slight injury in isolating eggs with needles or by group effects, that is, proximity of other eggs. In the group effects the regions nearest another egg or other eggs reduce more rapidly than other regions, evidently because oxygen decrease is more rapid there. The eggs incline to stick to glass; consequently, maintenance of uniform oxygen decrease by frequent change in position of eggs with the aid of gravity is usually not possible in the sealed preparation.

A slight ventrodorsal reduction differential has also been observed in hundreds of eggs, though not in all eggs seen from the side. While it seems to be characteristic, it is less distinct than the longitudinal gradient. What appears to be the characteristic course of dye reduction in the egg is indicated in Figures 12, 13, and 14. In some eggs the posterior polar region reduces a little more rapidly than adjoining, more anterior levels; but this gradient, when present, never extends far anteriorly and in most eggs does not appear. Whether it has any physiological significance is uncertain.

USE OF SODIUM HYDROSULPHITE

Rapid removal of oxygen from open Ringer-dye solution containing ovaries can be brought about by addition of very small amounts of sodium hydrosulphite, and reduction may be almost instantaneous or range in time from 1 to several minutes, according

to concentration of the agent. If it is not complete in a few minutes, further progress ceases because the agent in solution has been completely oxidized. Except when concentration of hydrosulphite is so high that reduction is virtually instantaneous, the reduction differential is the same as with gradual oxygen decrease by respiration of tissues in the sealed preparation. This course of reduction is very striking in the change from blue-green to red of Janus green, progressing over the whole ovary in a few seconds or in a minute or two, followed a little later by a similar progression of change from red to or toward colorless. As in the sealed preparation, the second color change may extend over the whole length in very young ovaries or may be limited to more apical levels in older ovaries, for example, the slender region in ovaries as in Figure 3. The occurrence and extent of this second stage of reduction depends on concentration of hydrosulphite as well as on stage of ovarian development. There may be gradual return of red with increase of oxygen in the open solution after the hydrosulphite is completely oxidized. After reduction of methylene blue with hydrosulphite there is also return of color, with increase of oxygen in the solution after complete oxidation of the hydrosulphite. With addition of a further minute amount of hydrosulphite the reduction gradient may be observed again.

The very minute quantities of sodium hydrosulphite used in this way for reduction are apparently not rapidly lethal or highly toxic. The ovaries retain their original form and appearance during reduction, even during repeated reductions with hydrosulphite concentrations low enough to produce gradual basipetal, rather than instantaneous, reduction. With instantaneous reduction death probably occurs very soon. After reduction of methylene blue in this way, the ovaries become somewhat yellowish or brownish, a change characteristic of ovaries killed by various other agents.

Use of hydrosulphite may be combined with sealing for rapid oxygen decrease and early reduction. Addition of hydrosulphite immediately before sealing in amount too small to decrease oxygen sufficiently to initiate reduction does lower oxygen content of the solution and bring about reduction earlier than it would otherwise occur. Used in sufficiently small amounts, the agent is a valuable aid in the study of differential dye reduction.

REDUCTION AND INJURY

The two oviducts unite a short distance basal to the ovaries; but for staining and reduction the two ovaries of a pair are separated, because when they remain side by side a mutual mass effect appears, reduction being most rapid in the region where each adjoins the other. When the two are separated, each retains a part of the oviduct with torn or cut end, or in some cases separation is in the basal region of the ovary itself. In all cases the torn end of the oviduct or the torn basal region of the ovary reduces early, almost or quite as early as the apical follicles, but reduction progresses only a short distance acropetally from the injured region. This basal reduction gradient is indicated in Figures 15, 16, and 19–21. The rapid reduction adjoining the cut or torn surface apparently results from activation following injury. In removal of ovaries the regions where the tracheae enter the ovaries are sometimes apparently injured by tension on the tracheae and reduce earlier than their level in the ovary would determine, again apparently in consequence of a stimulation or activation by the injury. Local injury of follicles or eggs apparently also induces early reduction locally. With sodium hydrosulphite the same effects of local injury appear.

MODIFYING AND COMPLICATING FACTORS

The effects of overstaining in altering the reduction differential have already been mentioned. Two factors, dye concentration and length of time that dye is present in the cells before reduction, are apparently concerned in this effect. If conditions provide for reduction within a few minutes after staining begins, relatively high dye concentrations may be used, for example, methylene blue 1/25,000, perhaps even higher, or Janus green 1/50,000. Long staining periods, even with concentrations much lower than these, appear to be more injurious than short periods. This is also true for embryonic echinoderm material (Child, 1936*a*, *b*) and for various other forms. There is apparently a progressive adsorption on, or union with, certain cell constituents after the dye enters the cell and an accompanying progressive injury. In some materials these constituents appear as deeply stained granules, and when they become visible in consequence of the deep stain the cell is injured and reduction is retarded. Echinoderm cleavage stages and blastulae show these granules very clearly. With overstaining, the nurse cells and oöcytes of more advanced follicles both become granular in appearance, and the more susceptible nurse cells may reduce later than the oöcyte or both may fail to reduce, particularly in earlier oöcyte stages.

Local mass effects on reduction of ovaries adjoining others have been mentioned. Under certain conditions mass effects may also appear in single ovaries and in groups of follicle strings not completely separated, that is, the great increase in mass of protoplasm basipetally may alter the course of reduction. If decrease of oxygen in the solution is slow, reduction may begin internally in the larger basal region or at some level below the apical tip in intact ovaries and in groups of parallel, partly isolated follicle strings. These modifications of reduction are most clearly eivdent in appearance of red with Janus green. With methylene blue, loss of color internally is obscured by the color of the outer parts of intact ovaries, but in partically separated follicle strings the beginning of reduction at some subapical level is readily observed.

In the young ovary of Figure 19, reduction begins internally in the subapical region; in Figure 20 it begins internally in the larger basal region; in Figure 21 two internal reduction centers appear, one subapical, the other in the large basal region. In these and other similar cases oxygen decrease in the external solution is not sufficiently rapid to bring about reduction externally before the internal decrease attains the reduction level. In the case of Figure 21 the higher oxidative activity at more apical levels apparently determines a reduction center subapically, and the larger mass in the basal region determines a second center there. With relatively long staining periods there may sometimes also be retardation of reduction in the more deeply stained superficial regions as compared with deeper parts. In ovaries slightly flattened between slide and cover glass after staining, the parts in contact with glass reduce earlier than those exposed to the solution. These modifications of the reduction differential and the extremely rich supply and ramification of tracheae suggest an intense oxidative activity, particularly in the younger ovaries.

Young ovaries like those in Figures 1–3, washed and kept in Ringer after moderate staining, show gradual decoloration, progressing basipetally. With methylene blue this decoloration appears exactly like differential reduction but takes place in open, well-aerated solutions. With Janus green there is merely loss of color without change to red. Under these conditions more or less of the apical region, often the apical third or half

of an ovary, may become completely colorless in 2–3 hours, but a color gradient increasing basipetally persists for a longer time, slowly fading to complete or almost complete decoloration.

Moreover, differential decoloration takes place, not only in Ringer after removal from dye solution, but in low concentrations of dye, for example, methylene blue 1/200,000 or 1/100,000, Janus green 1/400,000, or lower. In these concentrations staining is at first uniform or slightly deeper in the extreme apical region, but after 1–4 hours, depending on concentration and dye, decoloration becomes evident apically and gradually progresses basipetally. In the study of this decoloration, dye solutions were always used in large volume, 8–10 ml., and in many cases were completely renewed every 1–2 hours. With the large volume and frequent renewal of solution, progressive decrease in concentration by adsorption on the glass or otherwise is avoided. Nevertheless, in these dye solutions with large surface exposed to air and with frequent agitation the young ovaries and the young follicles of more advanced ovaries stain rapidly at first; but the more apical follicles, sometimes only the extreme apical 1 or 2, sometimes 3, 4, or 5, may become completely, or almost completely, colorless in the course of several hours with a color gradient increasing basipetally at lower levels. Little or no decoloration in dye concentrations that stain at first has been observed in advanced follicles and full-grown eggs. In still lower concentrations than those mentioned above, the more apical regions of ovaries stain scarcely at all and appear colorless very soon, and depth of staining increases basipetally in the younger parts of follicle strings.

If differential decoloration occurred only after removal from dye solution following staining, it might be regarded merely as differential loss of dye. It might be assumed that the cell constituents which hold the dye increase in relative amount basipetally in the ovaries; consequently, after return to Ringer following staining, the more apical regions lose dye more rapidly and more completely than those farther basal. It is, of course, possible that a loss of dye does take place under these conditions, but, since in all except extremely low concentrations apical regions stain as deeply as other parts and since differential decoloration also takes place in low dye concentrations, it seems evident that other factors than loss of dye must be concerned. Since the decoloration occurs in well-aerated solutions and since no visible trace of red appears in decoloration after staining with Janus green, it seems improbable that the decoloration is due to a gradual reduction of the very small amounts of dye present in the cells. The data suggest the possibility of a gradation, decreasing basipetally, in ability to decompose the dyes in some way with loss of color. After decoloration, the decolorized regions may be stained again in higher concentrations and may finally stain again after 12 hours or more in the same dye concentrations in which decoloration took place earlier. The torn end of the oviduct also undergoes decoloration in the low dye concentrations, but decoloration extends only a short distance from the injured region. Apparently, decoloration occurs while the ovaries are in good condition, and the ability to break down the dyes gradually decreases and disappears as death approaches. It is a familiar fact that various dyes gradually disappear from living cells, being apparently broken down in some way with loss of color. If the decoloration in the ovaries is a result of activities of living cells, as it appears to be, its progress basipetally is evidently an expression of the apicobasal differential in activity. However, the possibility that differential decoloration may be associated with approach of death requires consideration. It has not been possible to determine exactly when the isolated ovaries die. They retain their form and show little change in appearance, even

after 2 or 3 days in Ringer, though sooner or later the contents of the younger follicles become aggregated into irregular masses, and the ovaries gradually become yellowish or brownish in transmitted light. But the fact that differential decoloration in low dye concentrations occurs only during a few hours after isolation indicates that it is dependent on good physiological condition rather than on differential death or approaching death.

STAINING, REDUCTION, AND DECOLORATION AFTER LONG ISOLATION AND AFTER KILLING

Ovaries kept 20 hours in Ringer before staining show the same basipetal reduction differential with sodium hydrosulphite as do ovaries stained within a half-hour after isolation. Also they show the same basipetal decoloration in methylene blue 1/200,000 as do newly isolated ovaries. Young ovaries like those in Figures 1–3, placed in distilled water immediately after isolation, become almost or quite spherical and are apparently killed almost at once. After 20 hours in distilled water they stain rapidly and uniformly but less deeply than fresh ovaries in Ringer. In low dye concentrations they show no differential decoloration, but after return from dye to distilled water they decolorize very slowly with a slight basipetal differential, the loss of color progressing from the surface inward in each follicle. In the course of decoloration each follicle string appears as a series of stained regions increasing in size basipetally and more or less isolated from each other by colorless regions, from which the dye has diffused out. The stained regions are the deeper parts of each follicle. The basipetal gradient of loss of dye from these ovaries, which are unquestionably dead, is apparently determined largely or wholly by size of follicle. The smaller follicles with greater surface in relation to volume lose dye more rapidly than the larger. There may perhaps also be an increase in density or aggregation of follicle contents basipetally with corresponding decrease in rate of loss of dye.

Young ovaries, killed in boiling water, normal HCl, saturated $HgCl_2$, and 25 per cent formalin, become almost or completely spherical at once. In dye solutions after several washings they stain like distilled-water material, rapidly and uniformly but less deeply than living ovaries. They do not decolorize in low dye concentrations, but after return to water following staining there is gradual decoloration as in distilled-water material. With sodium hydrosulphite, after staining, reduction progresses basipetally and from the surface inward. This reduction differential in dead ovaries is, like the differential loss of dye, a consequence, at least in large part, of basipetal increase in follicle size. Reduction progresses inward in each follicle;and, with the basipetal increase in follicle volume in relation to surface, it is evident that unoxidized hydrosulphite will reach the deeper parts of the larger follicles less rapidly than those of the smaller. Even if rate of diffusion inward is the same in all follicles, a larger proportion of the agent diffusing in a given time will be oxidized in the larger than in the smaller follicles before reaching the deeper parts.

Here also, as in loss of dye, there may be a basipetal decrease in rate of diffusion. Dye reduction with hydrosulphite in these dead ovaries is well advanced or complete in the small apical follicles before it is evident even superficially in the basal follicles. It is perhaps unnecessary to note that such a differential is very different from a differential permeability in living cell masses, organs, or organisms. The living ovaries show no definite evidence of differential permeability except perhaps as between young follicles and full-grown eggs, and there the chorion rather than actual permeability of cell surfaces may be responsible.

CONCLUSION

The data speak for themselves and require little discussion. It is evident that a very definite and characteristic apicobasal dye-reduction pattern is present in the living ovary as a whole and in each follicle above a certain size. It appears highly probable that the physiological pattern of which the reduction gradient is an expression determines that the basal cell of each follicle shall become oöcyte, the other cells nurse cells, and the longitudinal gradient pattern of the full-grown egg develops as a part of the ovarian pattern. There is also evidence of a physiological differential from the outer ovarian surface inward, corresponding in direction to the ventrodorsality of the egg. The data do not constitute a demonstration that the axiate pattern of the egg is primarily a gradient pattern or that it is determined by the ovarian gradient pattern. They merely show a certain apparently complete correspondence or identity in certain features of the two patterns. Whether the egg pattern is primarily a dynamic gradient system or something else, it appears highly improbable that it originates independently of the ovarian pattern of cellular activity.

SUMMARY

1. Reduction of methylene blue and Janus green in modified Ringer solution of low oxygen content by developing ovaries of *D. hydei* shows a definite physiological ovarian pattern. Under properly controlled conditions, dye reduction in young ovaries progresses basipetally from the earliest follicle stages of the apical region and at any give level from the outer ovarian surface inward.

2. The reduction differentials may be altered or even reversed in direction by injury of the more susceptible apical regions through overstaining, by regionally unequal oxygen decrease in the solution, and by local injuries in isolation of ovaries.

3. Individual follicles above a certain small size show a basipetal reduction gradient. When nurse cells become distinguishable from the oöcyte, the basal cell of the follicle, the nurse cells reduce more rapidly than the oöcyte.

4. The longitudinal egg-axis coincides in direction with the longitudinal ovarian axis and gradient, and the apical or anterior pole of the egg is toward the apical ovarian pole. A longitudinal dye-reduction gradient coincident in direction with the ovarian gradient is usually visible in the full-grown egg. In all full-grown eggs, in which the relation of ventrodorsality to the ovarian pattern can be seen, the ventral side of the egg is toward the outer surface of the ovary, and there is apparently a slight ventrodorsal dye-reduction gradient in the eggs.

5. Sodium hydrosulphite may be used in minute amounts to decrease oxygen content in solutions containing ovaries and so to bring about rapid dye reduction. With sufficiently low concentrations the ovarian dye-reduction pattern, even in Ringer-dye solutions open to air, is identical with that resulting from decrease of oxygen by tissue respiration. With increasing concentrations of hydrosulphite, dye reduction is increasingly rapid until it becomes almost instantaneous.

6. In very low dye concentrations young ovaries, apparently in good condition, stain uniformly at first, but a decoloration gradient gradually appears, even with frequent renewal of dye solution in large volume, well aerated and remaining open to air. This gradient is identical in direction with the reduction gradient in low oxygen, but decoloration is usually complete only in the younger follicles constituting the apical third or half of an ovary. It is suggested that the more intensely active cells are or become able to

decompose the dye in low intracellular concentrations so that its color is lost and that this ability decreases basipetally from the most active to the less active levels.

7. Ovaries stained after killing by boiling water, HCl, $HgCl_2$, formalin, or distilled water show after return to water a gradual differential loss of dye and a differential reduction with hydrosulphite coincident in direction with the reduction pattern in living ovaries. These differentials in dead ovaries result largely or wholly from increase in follicle volume in relation to surface with increase in follicle size, though there may be a basipetal increase in density or aggregation of follicle contents in the dead ovaries. Diffusion of dye outward and of hydrosulphite inward is complete in the smaller, earlier than in the larger follicles.

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REGIONAL DIFFERENCES IN OXYGEN CONSUMPTION OF THE AMPHIBIAN GASTRULA

(Four text figures; one plate)

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THERE has been wide diversity in the results of investigations on the oxygen consumption of the amphibian organizer in the gastrula. Briefly, Brachet (1934), Brachet and Shapiro (1937), Fischer and Hartwig (1938), Brachet (1939), and Barth (1939) agree that the dorsal lip of the blastopore consumes oxygen at a higher rate than the opposite side of the egg. On the other hand, Waddington, Needham, and Brachet (1936) and Boell and Needham (1939) find that the dorsal lip respires at the same rate as the ventral ectoderm.

Now there are two main reasons why such disagreement might arise, for both the method of measurement of oxygen consumption and the preparation of the samples of tissue from the gastrula varied in the above experiments. Brachet (1934) arrived at his conclusion of a high O_2 consumption at the dorsal lip by removing parts of the gastrula and measuring the resulting decreased respiration. Brachet and Shapiro (1937), on the other hand, measured the oxygen consumption of the two halves of the gastrula by placing it intact in a glass tube of the same diameter. Thus the half containing the dorsal lip could be compared directly without injury with the half containing the ventral lip. Fischer and Hartwig actually cut out the dorsal lip and compared its O_2 uptake with pieces of the ventral ectoderm in the Warburg manometers. Waddington, Needham, and Brachet (1936) had previously used the method of explanting dorsal lip and ectodermal fragments and had used a microrespirometer. Boell and Needham (1939) explanted fragments into Cartesian divers. Brachet (1939) explanted the dorsal lip but compared it not with the ectoderm alone but with the corresponding tissue on the opposite side of the gastrula, i.e., the ventral lip.

In order to evaluate these results and plan the experiments recorded in this paper the organization of the early gastrula had to be carefully considered, especially in relation to stored materials such as yolk. Sections of the gastrula showed a gradient in the amount of yolk from the greatest amount at the vegetal pole to the least amount at the animal pole. The large macromeres can be seen filled with large yolk granules, while the much smaller micromeres have smaller yolk granules and relatively more cytoplasm in between. Bragg (1939) has illustrated the distribution of the yolk granules in various regions of the gastrula. There is relatively much more cytoplasm and many more nuclei in a sample of cells from the ectoderm (micromeres) as compared with the endoderm (macromeres).

Now the dorsal lip of the blastopore forms in the endoderm of the vegetal hemisphere and is partly composed of the large macromeres with much yolk and few nuclei, while the ectoderm, which is often used for comparison in respiration experiments, contains mostly cytoplasm and has many more nuclei. This comparison of respiration of the

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dorsal lip with ectoderm is confusing. For example, Waddington, Needham, and Brachet (1936) found that the O_2 consumption of their dorsal lip samples was a little higher than the ventral ectoderm. But, they add, the samples of dorsal lip were heavier because of the large yolk-laden cells present, so that on the basis of dry weight the rate of oxygen consumption was the same in both regions of the gastrula. However, the very fact which they observe in their paper, namely, that large yolk cells were present, means that the respiration of the dorsal lip must have been higher than the ventral ectoderm when corrected for the additional weight of the yolk. If the two regions use the same amount of oxygen in a given period of time, then, since the dorsal lip contains much more inert yolk, the protoplasm in this region must respire at a greater rate in order to consume the same amount of oxygen as the ventral ectoderm. It is clear (Fig. 1) that we must

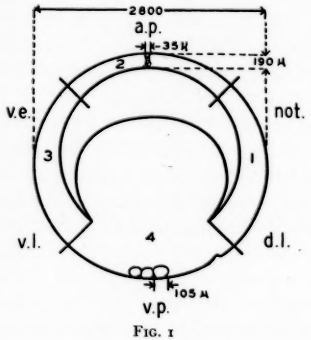

FIG. 1

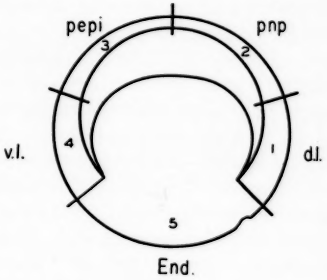

FIG. 2

FIG. 1.—Gastrula of *Amblystoma punctatum*, vertical section through the dorsal lip, showing regions used for measurements of oxygen consumption. *a.p.*, animal pole; *v.p.*, vegetal pole; *d.l.*, dorsal lip; *v.l.*, ventral lip; *not.*, notochord; *v.e.*, ventral ectoderm; Nos. 1, 2, 3, 4, refer to sectors of gastrula used for respiration studies. Dimensions are in micra.

FIG. 2.—Gastrula of *Amblystoma punctatum*. Vertical section through the dorsal lip showing regions used for measurements of oxygen consumption. See also Plate I. *d.l.*, dorsal lip; *pnp*, presumptive neural plate; *pepi*, presumptive epidermis; *v.l.*, ventral lip; *End.*, endoderm.

compare the dorsal lip with the corresponding region on the opposite side of the egg, where the same relative yolk content obtains; otherwise a high rate of oxygen consumption of the dorsal lip will be obscured by the relatively high yolk content, which adds to the dry weight or total nitrogen but does not consume oxygen.

This point cannot be emphasized enough, for it is a fundamental error that is found in all the measurements on the metabolism of the organizer except those by Brachet (1939), who gives figures showing the regions used for comparison with dorsal lip. In all other cases the organizer is compared with the ventral epidermis. This comparison is meaningless.

In order to avoid the errors pointed out in the preceding section the samples used for the determination were taken as in Figures 1 and 2 and Plate I. In Figure 1 the regions used are (1) dorsal lip, (2) animal pole, (3) ventral lip, and (4) vegetal pole; while in Figure 2 five regions are isolated: (1) dorsal lip, (2) presumptive neural plate, (3) presumptive epidermis, (4) ventral lip, and (5) endoderm. Plate I shows how the regions in Figure 2 are prepared. Thus, not only can the organizer be compared with the non-

organizer region of the opposite side of the egg, but the endoderm can be compared with the ectoderm, and a complete picture of the O_2 uptake of the various regions of the gastrula is obtained.

Table 9 records the rates of O_2 consumption in mm.^{3} of O_2 per hour per 100 mg. dry weight. It will be seen that the dorsal lip consistently uses O_2 at a greater rate than the ventral lip. Further, the gradient in rate of oxygen consumption which parallels the gradient in amount of yolk is clearly shown along the animal-vegetal axis by comparing the presumptive epidermis, the ventral lip, and the endoderm.

EXPERIMENTAL METHODS AND DATA

Briefly, the Winkler method for determination of dissolved oxygen was scaled to 230 mm.^{3} of water. This was possible by using a 250-mm.^{3} standard syringe for a respiratory chamber and transferring the water to a second 250-mm.^{3} syringe, to which Winkler's reagents were added. Finally, the titration with thiosulfate was carried out in a 300-mm.^{3} beaker, with the Linderstrom-Lang and Holter (1931) type of burette.

In detail Figure 3 shows the method of manipulation of the tissues and syringes. The syringe is first filled with Holtfreter's solution as in Figure 3, *a*. Next the tissues are drawn up into a curved pipette and allowed to fall into the filled syringe as in Figure 3, *b*. Solution is then expressed to the 300-mm.^{3} mark made on the syringe and the tip stoppered (Fig. 3, *c*). For shaking, the syringes are fastened to a rotating arm by means of fuse clips which are arranged at an angle of about 45° and rotated about one revolution every 45 seconds in a constant-temperature room (Fig. 3, *d*). At the end of the respiratory period, stopper is removed and 5 mm.^{3} expressed to clear dead space in tip (Fig. 3, *e*). Next a thin rubber washer *E*, which is perforated by a short glass tube, is inserted between the tips of syringe *O* and syringe *R*. The glass capillary running through the hole in the washer must be small enough to fit into the tips of the syringes (Fig. 3, *f*). With the two syringes held firmly together, the plunger of *O* can be pressed against a solid support and about 250 mm.^{3} of solution is forced from *O* into *R*, leaving the tissue behind in *O*. Syringe *R* is now ready for the reagents. Expel solution to 230-mm.^{3} mark and draw up 10 mm.^{3} of $MnCl_2$. Hold the syringe vertically and draw the contents of the tip into the barrel. Rotate syringe by twirling with fingers to mix. Wipe off tip with cleansing tissue and bring level of solution to tip of syringe again. Immerse tip in NaOH and KI and draw up 5 mm.^{3} Hold vertically and draw solution from tip into barrel. Wipe tip with cleansing tissue and rotate violently to mix solutions and to distribute precipitate evenly. Bring solution to tip of syringe again and cover with rubber cap (Fig. 3, *g*). Place syringe in horizontal position and let stand 4 minutes.

Next draw up 5 mm.^{3} concentrated phosphoric acid and rotate to mix until precipitate is dissolved. Place small droplet of starch in 300-mm.^{3} beaker and expel the 250 mm.^{3} from the syringe (Fig. 3, *h*). Place beaker in holder and titrate with 1/300 N thiosulfate. The procedure for the blank is exactly the same, and all the transfers are made as with the living tissue; three blank syringes are filled with the same solution of Holtfreter's as is used for the explants.

To empty syringe *O*, which contains the living tissue in about 30 mm.^{3} of Holtfreter's solution, immerse the syringe completely in large dish of Holtfreter's solution and draw out the plunger slowly (Fig. 3, *i*). The stopper is then placed in the tip of the syringe, and tissue may be removed by immersing the open end in Holtfreter's solution (Fig. 3, *j*).

The tissue is then rinsed in distilled water and placed in small platinum crucibles,

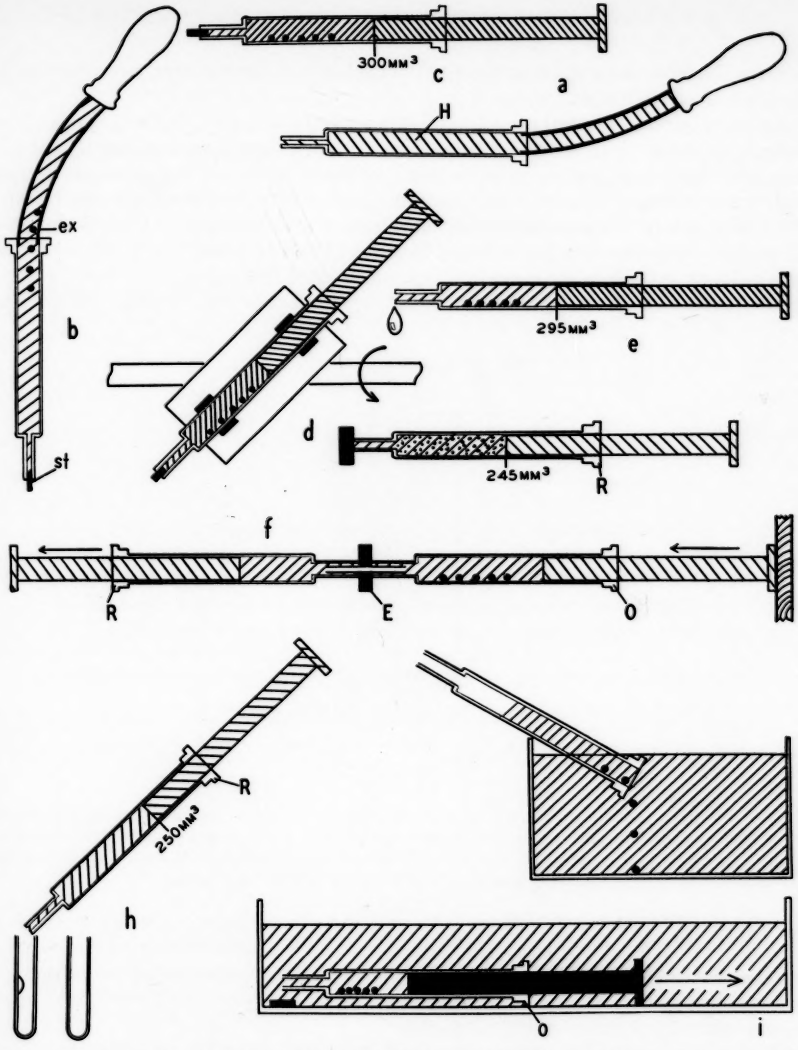

FIG. 3.—Figs. *a–j* show steps in the micro Winkler's method used. *ex*, explant; *st*, stopper; *H*, Holtfreter's solution; *E*, rubber stopper; *R*, reaction syringe; *O*, respiration syringe. *a*, filling of syringe from pipette; *b*, transfer of tissue to syringe; *c*, syringe with plunger at 300-mm.^{3} mark, stoppered during respiration; *d*, rotation of syringe causing explants to fall length of barrel. The angle can be adjusted for more or less movement of tissue. *e*, end of respiratory period, 5 mm.^{3} discarded; *f*, water transferred from respiratory syringe (*O*) to reaction syringe (*R*) by means of an airtight junction made of a piece of rubber, *E*, perforated by a glass tube which fits in the tips of the two syringes; *g*, Winkler's reagents are added by drawing them up into the reaction syringe and mixing by twirling the syringe rapidly with the finger and thumb; *h*, after phosphoric acid dissolves precipitate, the liberated iodine solution is transferred to a 300-mm.^{3} beaker with starch for titration; *i*, the respiratory syringe is refilled under water for removal of the tissue as in *j*.

dried, and weighed on a microbalance. Five explants of *Amblystoma punctatum* in these experiments weighed about 1 mg.

The microburette was adapted from Linderstrom-Lang and Holter (1931). A glass capillary (*g*) is bent into shape as in Figure 4, and mercury is used to displace the thiosulfate. It was found, after trying several methods of controlling the mercury, that simple screw clamps fastened firmly to the stand were suitable. One screw clamp (*a*) is used to regulate the amount of mercury from a reservoir and is kept closed all the time except when the burette is being cleaned. Another screw clamp (*b*) is used as a coarse adjustment, while the third (*c*) is used as a fine adjustment. The burette is easily read to 1 mm. of 1/300 N thiosulfate, which equals 0.0052 mm.^{3} of O_2. The oxygen in

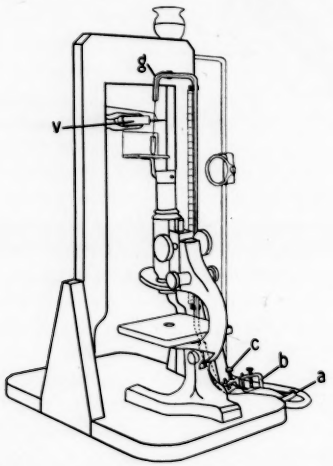

FIG. 4.—Microburette with vibrator for stirring solution. *g*, glass capillary burette connected to mercury reservoir by rubber tubing. *a*, *b*, *c*, three screw clamps. *b* and *c* used to deliver thiosulfate from burette. *V*, vibrator which transmits vibrations to burette tip by means of thread. The microscope is simply used as a rack and pinion for raising and lowering the beaker for titration.

the 300 mm.^{3} of Holtfreter's is about 1.7 mm.^{3} and gives a titration value of about 240 mm. of 1/300 N thiosulfate when 230 mm.^{3} is used for a sample. Since only 230 mm.^{3} of solution are used for the determination of oxygen, the conversion factor for oxygen consumed by the tissue in 300 mm.^{3} of solution is $0.0052 \times 30/23 = 0.00675$ mm.^{3} of oxygen for every millimeter of thiosulfate used.

At first an electromagnetic stirrer was used, but it was found to be more cumbersome than a vibrator. The vibrator is an inexpensive electric shaver (doorbell vibrator also works), which transmits its vibrations to the capillary tip of the microburette by means of a thread. This is an extremely effective stirrer and is best controlled with a foot push button, which leaves the hands free to adjust the screw clamps and the microscope. When dead spaces occur, the beaker can be shifted up or down by the microscope rack and pinion. It is seen that, although the titrations are easily reproducible (Table 1), the respiration measurements on the eggs are more variable (Table 2). This led to a survey of the factors influencing respiration in syringes. Agitation of the eggs in the syringes

TABLE 1

THE REPRODUCIBILITY OF THE TITRATIONS

In the following experiment the syringe is filled with 230 mm.³ of Holtfreter's solution, the reagents added, and the syringe emptied into a 300-mm.³ beaker and titrated with 1/300 N sodium thiosulfate. 1 mm. of thiosulfate in burette = 0.278 (volume of 1 mm. of burette) × 0.00333 (normality of thiosulfate) × 5.6 mm.³ O_2 (oxygen equivalent of thiosulfate) = 0.0052 mm.³ O_2. An experimental error of about 0.03 mm.³ O_2 is indicated.

Titration No.	Mm. Thiosulfate	Mm. Thiosulfate × 0.0052 Mm.³ O_2	Titration No.	Mm. Thiosulfate	Mm. Thiosulfate × 0.0052 Mm.³ O_2
1	227	1.18	6	225	1.17
2	225	1.17	7	225	1.17
3	227	1.18	8	225	1.17
4	224	1.16	9	224	1.16
5	229	1.19			

TABLE 2

THE REPRODUCIBILITY OF THE RESPIRATION MEASUREMENTS

Two eggs in Stage 9 were placed in each of three syringes in 300 mm.³ of Holtfreter's solution; 230 mm.³ of solution was used for a Winkler's determination. Conversion factor is therefore 0.0052 × 300/230 = 0.00675 for 1 mm. of burette.

The differences in rate could be accounted for by an experimental error of 0.03 mm.³ O_2 in the titration.

Syringe	Thiosulfate (Mm.)	Blank-Exper. (Mm.)	Mm.³ O_2	Time (Hours)	Mm.³ O_2/Hour/Egg
1	158	77	0.52	2.1	0.124
2	137	98	0.66	2.43	0.136
3	133	102	0.69	2.7	0.128
Blank	235	.....	.....	.....	.....

TABLE 3

THE EFFECT OF AGITATION ON RESPIRATION OF EGGS IN SYRINGES

One egg in a syringe. One syringe was rotated constantly, keeping the egg in motion, while another was stationary at the same temperature. Rate = mm.³ O_2/hour/egg.

Experiment	Stage	Syringes Rotated			Syringes Stationary			Increase Due to Rotation (Per Cent)
		Mm.³ O_2	Hours	Rate	Mm.³ O_2	Hours	Rate	
1	10–11	0.58	3.48	0.167	0.41	3.62	0.113	47
2	10–11	0.81	5.28	0.154	0.64	5.8	0.110	40
3	9	0.61	5.73	.....	0.47	5.9	.....	35
		0.66	5.15	0.116	0.57	6.0	0.086	
		0.69	5.50	.....	.....	.....	.....	

is a factor that brings about a 40 per cent increase in oxygen consumption over that of stationary eggs (Table 3). Since rather large numbers of cells are used for respiration measurements, it was thought that size might have an influence on rate of oxygen consumption following surface-volume relationships. Little or no difference is found when the surface-volume relationship is changed by a factor of 2 (Table 4). Next the general

TABLE 4

EFFECT OF SIZE OF EXPLANT ON RATE OF RESPIRATION

Strips of ectoderm cut out of gastrula; one half fused in pairs, while other half remains single. Fusion in pairs approximately halves the amount of surface compared with singles. Rate = mm.^{3} O_2 per hour for same volume of explant. The differences are within the experimental error of 0.03 mm.^{3} O_2 for the titrations.

Experiment	3 Fused Pairs			6 Singles			Fused − Single / Single
	Mm.^{3} O_2	Hours	Rate	Mm.^{3} O_2	Hours	Rate	(Per Cent)
1	0.90	3.25	0.28	0.88	3.37	0.26	8
2	0.47	2.47	0.19	0.44	2.67	0.17	14

TABLE 5

COMPARISON OF THE RESPIRATION OF THE WHOLE GASTRULA WITH THE SUM (Σ) OF ITS PARTS

Gastrula cut into six parts; all six parts placed in one syringe, while the whole gastrula is placed in another syringe for comparison. Rate = mm.^{3} O_2 per hour per gastrula.

Experiment	Remarks	Whole Gastrula			Σ of 6 Parts			Σ6 − Whole / Whole
		Mm.^{3} O_2	Hours	Rate	Mm.^{3} O_2	Hours	Rate	(Per Cent)
1	Two gastrulae in each syringe, measured 1 hour after cutting	0.52 0.66 0.69	2.1 2.43 2.7	0.13	0.62 0.84 0.71	2.35 2.33 2.54	0.15	+15
2	One gastrula in each syringe, measured 24 hours after cutting. Syringes rotated	0.81	5.28	0.15	0.64	5.08	0.13	−18
3	One gastrula in each syringe, measured 24 hours after cutting. Syringe stationary	0.64	5.8	0.11	0.47	5.65	0.08	−24

effect of injury by cutting and recovery from injury was determined by comparing the oxygen consumption of the whole gastrula with that of the sum of its parts. Table 5 indicates that there may be a slight stimulation of oxygen consumption due to cutting, but 24 hours later the explants consume oxygen at a lower rate than the whole egg. However, the differences between the respiration of the whole gastrula and the sum of its parts may not be significant, and we can say only that cutting the gastrula into six parts

has little effect on the rate of oxygen consumption. This observation is very important since conceivably the injury of cutting and the additional surface offered in a cut fragment might cause a great change in the rate of oxygen consumption over that of the same fragment *in situ*. In this connection it should be pointed out that the explanted parts of the gastrula do not develop into the same structures that they would have in the intact gastrula. Therefore, differences in O_2 consumption are to be expected. Finally, in measuring the respiration of the endoderm a few cells were often carried over from the respiration syringe into the reaction syringe, and this might introduce an error into the titration. Table 6 shows that large fragments of yolk are necessary before any appreciable change in the titration for oxygen is detected, so that no correction is necessary

TABLE 6

EFFECT OF YOLK CELLS ON TITRATION FOR OXYGEN

A fragment from the vegetal hemisphere was drawn up into a syringe and shaken until a suspension of yolk cells in Holtfreter's solution was obtained. Winkler's reagents were then added and titration made. The figures are mm.^{3} O_2 in 230 mm.^{3} solution.

The "small fragment" gave about the same amount of suspended matter that is usually found in the syringe when fragments of endoderm (No. 4, Fig. 1) are used for respiration. A few cells become detached and are carried over into the reaction syringe but, as shown above, do not interfere with the titration. However, when much larger amounts of suspended matter are present, a correction is necessary. In the experiments recorded in this paper no corrections were necessary.

Holtfreter's Clear	Holtfreter's + Small Fragment	Holtfreter's + Large Fragment	Holtfreter's Clear	Holtfreter's + Small Fragment	Holtfreter's + Large Fragment
1.30	1.30	1.22	1.30	.....	1.25
1.30	.....	1.23	1.30	.....	.....
1.30	.....	1.22			

for small amounts of endoderm cells. Since in our experiments only very small fragments were carried over into the reaction syringe, it is clear that they would cause no error. In the case of tissues other than endoderm, cells did not dissociate from the explant and there was no possible contamination of the sample of solution for the Winkler's test.

RELATIVE RATES OF RESPIRATION OF EXPLANTS OF THE EARLY GASTRULA

The eggs of *A. punctatum* (*A. maculatum*), *A. opacum*, and *Rana pipiens* were used in the experiments. The explants were prepared in different ways so as to compare different regions of the gastrula. In some cases the roof of the blastocoel was divided into four parts (Fig. 2, Pl. I), in other cases into three parts (Fig. 1). Plate I, *A*, shows how the gastrula is cut open; I, *B* and *C*, cutting and healing of explants. In one experiment the amount of large yolk cells included in the dorsal- and ventral-lip samples was purposely varied to show the effect of yolk on O_2 uptake of these regions.

I. THE EFFECT OF VARIATIONS IN SAMPLING ON THE RELATIVE O_2 CONSUMPTION OF THE PARTS OF THE GASTRULA

Since the distribution of the yolk is a very important factor in the rate of respiration of parts of the gastrula, a series of five determinations of O_2 consumption was made, in

which the amount of yolk included in the samples varied. The gastrulae, Stage 9 of *A. opacum*, were prepared as in Plate I, *A*, by means of two lateral incisions on either side of the dorsal lip, followed by transverse cuts at the dorsal lip and at the ventral lip. The yolk mass is then removed, and the remaining tissue is trimmed and divided into three approximately equal parts: (1) dorsal lip, (2) ectoderm from animal pole, and (3) ventral lip (Fig. 1). Piece No. 4 is the large endodermal mass of cells and No. 6 the remainder of the gastrula which results from trimming the sides of the blastocoel before cutting into three equal parts. Number 6 consists of the two small pieces trimmed off the sides of the flattened roof of the blastocoel.

In preparing pieces No. 1 and No. 3 in one case there were no yolk cells adhering to the tissue, while in another case a large number of yolk cells were included. In the remaining three experiments a variable amount of yolk cells was present in pieces No. 1 and No. 3. Number 2, which comes from the animal pole, is, of course, always free of yolk cells. Number 4 is composed exclusively of yolk cells, and No. 6 may have a variable amount.

TABLE 7

EFFECT OF VARIATIONS IN THE SAMPLES OF TISSUE UPON RATE OF O_2 CONSUMPTION

Temperature 18°.5±0°.1 C. Mm.^{3} of O_2/hour/100 mg. dry weight

Experiment	Description	1	2	3	4	6
		Dorsal Lip	Animal Pole	Ventral Lip	Endoderm	Remainder
1.....	No yolk cells adhering to Nos. 1, 2, 3, and 6	14.5	25.0	8.6	2.7	16.7
2.....	Large amount of yolk cells adhering to Nos. 1, 3, and 6	7.2	18.8	3.5	2.8	6.3
3.....	Some yolk cells adhering to Nos. 1 and 3	8.4	14.5	7.1	1.75	13
4.....	Some yolk cells adhering to Nos. 1 and 3	9.3	18.0	6.7	2.4	.....
5.....	Some yolk cells adhering to Nos. 1 and 3*	6.5	12.8	5.7	2.1	9.3

* Rates unusually low—eggs had been kept at 5° since cleavage.

These parts heal very rapidly in *A. opacum* and can be transferred from operating dish to the syringes in 1–2 hours after cutting. The syringes are sealed and placed in a water bath during the period of respiration. The results in Table 7 show how variation in the amount of yolk can influence respiration. Thus in Experiment 1, where the dorsal lip is free of adhering yolk cells, it uses oxygen at a rate of 14.5 mm.^{3} per hour per 100 mg. dry weight, while in Experiment 2 the rate is only 7.2 when large amounts of yolk cells are present.

Experiments 3, 4, and 5 show variations which are in part due to variations in yolk and in part to technique. In general the dorsal lip respires at a greater rate than the ventral lip, and the animal pole at a much greater rate than the endoderm of the vegetal pole. The order of the rates of respiration of the parts may be summarized thus: Animal pole > dorsal lip > ventral lip > endoderm.

II. COMPARISON OF OXYGEN CONSUMPTION OF THE DORSAL LIP, THE ANIMAL POLE, AND THE VENTRAL LIP OF THE EARLY GASTRULA

The respiration of the parts of the egg of *A. punctatum* and *R. pipiens* was measured, and to reduce variability the pieces were cut out so that no adhering yolk cells were present (see Fig. 1 for regions used). Of course, since the ventral and dorsal lip are in the intermediate zone between the micromeres and the macromeres, the explants always contain some yolk-filled cells.

TABLE 8

OXYGEN CONSUMPTION OF PARTS OF THE GASTRULA

Rate = mm.³ of oxygen per hour per 100 mg. dry weight. Five pieces of each region placed in one syringe.

Description of Part		Weight in Milligrams	Mm.³ of Oxygen	Time in Hours	Rate	Remarks
No.	Region					
1.....	Dorsal lip	0.884	0.378	1.91	22.4	Temp. 20°. *A. punctatum.* Syringes rotated. Three determinations on same tissues
			0.497	2.91	19.3	
			0.370	2.26	18.5	
2.....	Ectoderm	0.793	0.452	2.45	23.3	
			0.527	2.88	23.1	
			0.445	2.00	28.0	
3.....	Ventral lip	1.404	0.426	2.20	13.8	
			0.586	2.88	14.5	
			0.344	1.78	13.8	
1.....	Dorsal lip	1.211	1.07	2.63	33.6	Temp. 21°. *A. punctatum.* Syringes rotated. Two determinations on same tissues
			0.852	2.23	31.6	
2.....	Ectoderm	1.054	0.686	2.38	27.4	
			0.802	2.73	27.9	
3.....	Ventral lip	1.516	0.890	2.86	20.6	
			0.776	2.48	20.6	
1.....	Dorsal lip	1.301	0.715	2.36	23.2	Temp. 18°.5. *A. punctatum.* Syringes rotated
2.....	Ectoderm	1.339	0.686	1.98	26.0	
3.....	Ventral lip	1.520	0.470	2.08	15.4	
1.....	Dorsal lip	0.652	0.575	3.83	23.0	Temp. 19°.5. *R. pipiens.* Syringes not rotated
2.....	Ectoderm	0.688	0.775	4.02	28.0	
3.....	Ventral lip	1.010	0.800	4.20	18.9	
4.....	Endoderm	1.953	0.700	4.67	7.7	

The results (Table 8) bear out the conclusions of the previous section, namely, (1) that there is an animal-vegetal gradient in the rate of respiration and (2) that the dorsal lip respires at a greater rate than the ventral lip.

III. COMPARISON OF DORSAL LIP WITH PRESUMPTIVE NEURAL PLATE, PRESUMPTIVE EPIDERMIS, AND VENTRAL LIP OF THE EARLY GASTRULA

In these experiments the roof of the blastocoel is cut into four approximately equal pieces along the anteroposterior axis. Two lateral incisions are made on either side of the dorsal lip, followed by two transverse cuts, one at the dorsal lip and one at the ventral

lip. This completely separates the mass of yolk-filled cells from the remainder, which is flattened out as in Plate I, *A*. This strip of tissue is trimmed and then cut in four equal parts, as in Plate I, *B*, and Figure 2. These pieces remain in the operating dish until they begin to curl up and heal, as in Plate I, *C*. The debris is then washed off and the explants transferred to separate syringes by means of a curved pipette (Fig. 3, *b*). In the syringes they complete the process of healing into spherical masses, which continue to differentiate. The syringes are placed on a revolving drum at an angle of about 45° to horizontal axis, and the explants fall through the length of the syringe about twice every minute. This mixes the water and does not injure the explants, except the endoderm, as they fall slowly. The endoderm explants cytolyze when rotated and hence were measured under stationary conditions.

TABLE 9

OXYGEN CONSUMPTION OF THE PARTS OF THE GASTRULA

Rate = mm.³ of oxygen per hour per 100 mg. dry weight. See Figure 2 for parts used. Five pieces of each part in one syringe.

Description of Part		Weight in Milligrams	Mm.³ of Oxygen	Time in Hours	Rate	Remarks
No.	Region					
1	Dorsal lip	1.071	0.427	2.08	19.2	Temperature, 19°. *A. punctatum*. Syringes rotated
2	Presumptive neural plate	1.807	0.402	2.44	20.4	
3	Presumptive epidermis	1.009	0.478	2.61	18.2	
4	Ventral lip	1.449	0.427	2.83	10.4	
1	Dorsal lip	1.119	0.548	2.33	21.4	Temperature, 19°5. *A. punctatum*. Syringes rotated
2	Presumptive neural plate	0.684	0.471	3.02	23.8	
3	Presumptive epidermis	0.812	0.471	2.79	20.1	
4	Ventral lip	1.197	0.420	2.56	13.7	
1	Dorsal lip	0.846	0.460	3.22	16.9	Temperature, 19°5. *R. pipiens*. Syringes not rotated
2	Presumptive neural plate	0.576	0.560	3.38	28.6	
3	Presumptive epidermis	0.809	0.610	3.55	21.2	
4	Ventral lip	1.297	0.510	3.82	10.3	
5	Endoderm	2.395	0.390	3.99	4.1	

By this means the oxygen consumption of four different regions of the gastrula is determined. These four regions are shown in Figure 2 and correspond roughly to (1) the dorsal lip, i.e., presumptive notochord and mesoderm; (2) presumptive neural plate; (3) presumptive epidermis; (4) ventral lip, i.e., posterior ectoderm and tail mesoderm. In preparing samples, Nos. 1 and 4 were chosen so as to contain the same amount of light-colored, yolk-filled cells.

Table 9 shows that the order of rates of respiration of explants is as follows: First experiment, presumptive neural plate > dorsal lip > presumptive epidermis > ventral lip. Second experiment, same order. Third experiment, presumptive neural plate > presumptive epidermis > dorsal lip > ventral lip.

THE YOLK CONTENT OF VARIOUS REGIONS OF THE GASTRULA

It is well known that there is a gradient in the amount of yolk in the egg with the highest concentration at the vegetal pole and lowest at the animal pole. During cell division this yolk becomes segregated into the cells of the early gastrula and forms a part of the

dry weight or nitrogen of fragments used for oxygen consumption or chemical analysis. In order to determine the relative activity of the protoplasm of parts of the gastrula it is necessary to know how much yolk is present in each part.

There are two ways of attacking this problem. First, it is possible to make histological sections and determine the relative amount of yolk granules by measuring them with an ocular micrometer. Second, since the yolk has a high specific gravity, some measure of its concentration in various regions can be arrived at by determining the specific gravity of fragments. Both of these methods were used.

I. THE SPECIFIC GRAVITY OF FRAGMENTS OF THE GASTRULA

The gastrula of *R. pipiens* and *A. punctatum* are cut into six fragments, as in Table 4. Number 1 is a piece from the dorsal-lip region; No. 3 is from the animal pole; and No. 6 is a sample of yolk-laden cells of the endoderm.

The specific gravity of these fragments was found by suspending them in gum arabic and determining the specific gravity of a solution in which the fragments remained stationary. Next, the specific gravity of the yolk granules was determined by crushing the cells and centrifuging in solutions of sucrose. This gave a value of 1.2585 for the granules. In order to calculate the percentage of yolk in the fragments whose specific gravity had been determined, it was necessary to arrive at some value for the specific gravity of yolk-free protoplasm. This was done by taking tissues from the swimming tadpole and centrifuging in gum arabic solutions. The heart, tail, and liver were used, all giving a value of about 1.023. From these values the percentage of yolk has been calculated on the basis of both volume and weight and is given in Table 10.

We see, then, that the amount of yolk varies from 24.5 per cent to 49 per cent from the animal to the vegetal pole of the gastrula of *R. pipiens* and 24 to 51 per cent in *A. punctatum*. The difference between animal and vegetal pole may be even greater if smaller fragments of tissue are used. These calculations are made on the assumption that the yolk granules and the yolk-free protoplasm make up the bulk of the cells.

II. DETERMINATION OF YOLK CONTENT BY HISTOLOGICAL METHOD

The above calculations depend on a not too accurate determination of the specific gravity of yolk-free protoplasm, and so it is necessary to compare with a totally different method. Bragg (1939) has made a study of the yolk distribution in several amphibian eggs. From his figures (p. 275, Figs. 67, 68) it is possible to calculate the relative amount of yolk in the animal pole as compared with the vegetal pole. In *Triturus torosus* it is found that 19.7 per cent of the content of the animal pole is yolk, while at the vegetal pole 58 per cent is yolk. Similar results can be calculated from *Bufo cognatus*, where drawings were made by Bragg of the cells from the animal pole (micromeres), the vegetal pole (macromeres), and an intermediate zone. While no such careful histological examination of *A. punctatum* and *R. pipiens* has been made, gross examination of the sections of the gastrula shows a similar yolk distribution.

The agreement of two totally independent calculations of the yolk content makes it certain that a gradient in the concentration of yolk from at least 20 per cent in ectoderm to 60 per cent in endoderm exists in the amphibian gastrula. Naturally, these figures will vary as the relative amount of yolk varies a great deal in various species of amphibia. *A. punctatum* is a very large egg and probably shows a greater difference in yolk concentration than some other forms.

THE ABSOLUTE RATE OF RESPIRATION OF EXPLANTS OF EARLY GASTRULA

Although the fragments used for the measurement of oxygen consumption and those used for the specific-gravity determination are not strictly comparable with regard to size and so with regard to yolk content, it seems valuable to give an approximation of the values of O_2 consumption for yolk-free protoplasm. This has been calculated for three sets of data in Table 5. Twenty per cent yolk is a reasonable figure for the ectoderm near the animal pole; 30 per cent for the dorsal-lip region is only a rough value because

TABLE 10

SPECIFIC GRAVITY AND YOLK CONTENT OF FRAGMENTS OF THE GASTRULA OF *R. pipiens* AS TESTED BY GUM ARABIC

Fragment Number	Region	Specific Gravity	Per Cent Yolk Calculated as Yolk = 1.2585, Protoplasm = 1.023	
			Volume	Weight
3	Ectoderm animal pole	1.0725	21.0	24.5
4	Ventral ectoderm	1.0775	23.1	27.0
5	Ventral lip	1.0900	28.4	32.7
6	Endoderm	1.1280	44.0	49.0

SPECIFIC GRAVITY AND YOLK CONTENT OF FRAGMENTS OF THE GASTRULA OF *A. punctatum* AS TESTED WITH GUM ARABIC

Fragment Number	Region	Specific Gravity		Per Cent Yolk Calculated as Yolk = 1.2585, Protoplasm = 1.023	
		Expt. 1	Expt. 2	Volume	Weight
1	Dorsal lip	1.092	1.092	30	34
2	Ectoderm and notochord	1.077	1.077	23	27
3	Ectoderm (animal pole)	1.071	1.071	20	24
4	Ectoderm (ventral)	1.076	1.075	22	26
5	Ventral lip	1.093	1.092	30	34
6	Endoderm	1.130	1.132	46	51

this region can vary a great deal in amount of yolk, being situated, as it is, at the border between the animal and the vegetal halves. This value is probably correct for *R. pipiens* and *A. punctatum* but is too low for *A. opacum*, where the dorsal- and ventral-lip fragments used for respiration contained relatively more yolk cells. Probably 40 per cent would be a better value for the latter, and in Table 11 both 30 per cent and 40 per cent were used. Fifty per cent for the endoderm is reasonable, as the fragments used for the measurements of oxygen consumption and specific gravity were about the same.

Using these values, the O_2 consumption of yolk-free protoplasm is calculated and found to vary from 5 to 22.5, or a 4*x* gradient, in *A. opacum*, and from 8 to 35, likewise a

4x gradient, in *R. pipiens*. The dorsal lip in the case of *R. pipiens* and *A. punctatum* appears to respire at about the same rate as the ectoderm but at a decidedly higher rate than the ventral lip.

It would appear, then, that there is a steep gradient in oxidative activity of the protoplasm of the egg on the ventral side of the gastrula, while the dorsal side is almost uniform. Further determinations of O_2 consumption of smaller and well-localized fragments is absolutely necessary before the exact picture of the dorsal side of the egg is complete.

TABLE 11

CALCULATIONS OF THE O_2 CONSUMPTION OF YOLK-FREE PROTOPLASM OF VARIOUS REGIONS OF THE GASTRULA*

FRAGMENT	OXYGEN CONSUMPTION			PER CENT YOLK BY WEIGHT	OXYGEN CONSUMPTION CORRECTED		
	*R. p.**	*A. o.**	*A. p.**		*R. p.*	*A. o.*	*A. p.*
Ectoderm	28	18	26	20	35	22.5	32.5
Dorsal lip	23	9†	23	30 (40)	33	13(15)	33
Ventral lip	19	6†	15	30 (40)	27	8.6(10)	21
Endoderm	4	2.5	.....	50	8	5	.....

* *R. p.* = *Rana pipiens*; *A. o.* = *Amblystoma opacum*; *A. p.* = *A. punctatum*.

† Relatively more yolk cells than in *R. p.* and *A. p.*, so 40 per-cent of yolk used.

REGIONAL DIFFERENCES IN CELL SIZE IN GASTRULA

Aside from the distribution of yolk, the cells vary in size, and so regions vary in the number of nuclei per unit volume of weight. Figure 1 gives the dimensions of a living gastrula of *A. punctatum* (Stage 9, Harrison), the thickness of the roof of the blastocoel, and the average diameter of cells from the animal pole and the vegetal pole. It is seen that the ectoderm cells average about one-third the diameter or are about one twenty-seventh as large as the endoderm cells.

Whether this size factor has any effect on O_2 consumption is difficult to say. In the section on method (Table 4) I found little difference between the respiration of small and large fragments of the gastrula, although the size ratio was not nearly so high as 1:27. The density of the nuclei would show the same ratio and might have an effect on the rate of respiration.

Finally, it should be pointed out that there may be a difference in O_2 tension in the cells of the vegetal as compared with the animal pole. Since the rate is so low in the cells of the vegetal region, less O_2 is used up as it diffuses in from the medium, and the concentration of O_2 in the cells under the surface would be higher than in the animal regions, where the greater rate would lower the O_2 tension much more. Thus cells situated deep inside a mass of ectoderm may be under partially anaerobic conditions, while this is less likely in the case of the endoderm. An experiment recorded under the section on methods (Table 3) shows that shaking may cause an increase of 40 per cent in rate of respiration over stationary eggs, which indicates that the rate of diffusion of O_2 to the deeper-lying cells is not so fast as the rate of respiration, resulting in an O_2 deficiency.

DISCUSSION

It is clear from the data that an important factor in rate of respiration is the distribution of the yolk in the early gastrula. While it is a relatively simple problem to de-

termine the amount of oxygen used by any particular region of the gastrula, this does not tell us much about the rate of oxygen consumption of the protoplasm of this region.

If we turn to the "corrected" values (Table 11) for rate of oxygen uptake, we find that the ventral half of the gastrula has a region of high respiration in the animal hemisphere, lower in the intermediate zone of the ventral lip, and lowest in the vegetal hemisphere. Thus a gradient of respiration exists even after the correction is made for yolk content. On the dorsal side, however, the picture is quite different. The animal pole respires highest. The intermediate zone of the dorsal lip respires at about the same rate, and then there is a sudden drop in rate in the vegetal hemisphere below the dorsal lip.

Thus there are definitely two regions in the gastrula where the protoplasm is very active in respiration: one at the animal pole and one in the vicinity of the dorsal lip. Since we have no measurements of any regions between these two, it is not possible to say whether the active regions are contiguous or not. This correlates well with the findings of Bellamy (1919), who used $HgCl_2$ for susceptibility, with Piepho (1938), who used reduction of brilliant cresyl blue, and Fischer and Hartwig (1936), who used reduction of other dyes.

With regard to the gradients of respiratory rate, two possibilities should be made clear. First, the gradient is probably much steeper than indicated by the measurements in this paper. Rather large samples of tissue from the animal hemisphere were used, and there is every reason to believe that, if very small pieces had been cut out of the animal pole, much higher rates of O_2 uptake would have been observed—that is, the larger the sample, the more of the low-respiring tissue from the intermediate zone is included and the lower the average rate. At the opposite end of the gradient it is also possible that, with the use of smaller samples from the endoderm, lower rates would be obtained.

Second, since regions from the animal pole and from the dorsal lip respire at the highest rates, there may be a region in between which has a lower rate, thus establishing a double gradient. Of course, it is possible that the rates are the same throughout the whole dorsal region. This could be determined only by taking a strip of tissue from the dorsal lip to the animal pole and dividing it into at least three parts.

The high rate of O_2 consumption in the dorsal-lip region may be significant in more than one respect. First of all, it may be the result of the increased activity of the dorsal lip in gastrulation, i.e., increased cell division and migration of cells to form the roof of the archenteron, while the opposite side does very little in the way of involution during gastrulation. Second, it may result from the specific metabolic processes accompanying the differentiation of the notochord and the mesoderm, i.e., this dorsal-lip region is different from the opposite side of the gastrula in that it has a different presumptive value. Third, the high O_2 consumption may relate with the organizer action during gastrulation, i.e., the dorsal lip may liberate certain products of oxidation which stimulate the neural plate in the overlying ectoderm.

The third interpretation is an interesting one in view of recent developments in the studies of the organizer. The writer (Barth, 1941) has found that the presumptive neural plate will self-differentiate under certain conditions without contact with the organizer. Further, it has been found by a number of investigators that several chemically different substances will stimulate the ectoderm to form a neural plate. Therefore, the conditions are present in the early gastrula for a very simple relationship between organizer and presumptive neural plate. All we need to have is almost any substance liberated by the

organizer cells acting upon a labile determination in the presumptive neural plate. It is certainly quite possible that the substance might be a product of metabolism, such as urea, ammonia, lactic acid, or perhaps even CO_2.

In a series of studies on the organization of the hydranth in *Tubularia* (Barth, 1940) it was shown that O_2 may act as an organizer. Child (1928) had previously shown that the hydranth forms as a result of differential exposure of tissues to the environment, i.e., where free exchange of O_2 and excretions occurs, the hydranth will differentiate. Not only does O_2 stimulate the formation of a hydranth but it actually reverses the normal polarity of stem, so that the distal part of the hydranth always forms in relation to the incoming supply of oxygen (Zwilling, 1939). The explanation of the effect of O_2 is through an increase in O_2 consumption with increase in O_2 tension, resulting in a gradient of respiratory metabolism (Child, 1929; Barth, 1940).

There are certain similarities in amphibian organization. The animal pole of the gastrula exhibits the highest rate of O_2 consumption and forms head ectodermal structures. Dorsal lip is a region of high O_2 consumption and forms the mesodermal and endodermal structures of the head region. In comparison with hydroid organization it is apparent that in both cases the stimulus for organization proceeds from tissues exhibiting a high rate of oxygen consumption.

COMPARISON WITH OTHER WORK

While it is very difficult to compare any of the work on respiration of the organizer because investigators have not been clear as to just what cells were used, a few agreements may be pointed out.

In the first place, Fischer and Hartwig find that the dorsal lip of *A. mexicanum* consumes O_2 at a rate of 21.3 and the ectoderm at 19.2. I find that the values are 21.4 and 20.1. The agreement is more fancied than real, however, if one considers that the species were different, as were the methods of measurement and possibly the actual samples of tissue.

Second, Brachet and Shapiro find that the oxygen consumption of the side of the egg containing the dorsal lip was about 47 per cent higher than the opposite side. In calculating the sum of the oxygen consumption of the individual parts from the two sides of the egg, I found the value to be 42 per cent higher.

Brachet (1939), in comparing rather large samples of the egg of *R. fusca*, found that the dorsal lip respired 31 per cent higher than the opposite side of the gastrula. In one of my experiments with *R. pipiens* where samples were large the value was 22 per cent; in another where the samples were small the value was 64 per cent.

It is not possible to compare results with those of Waddington, Needham, and Brachet (1936) and with Boell and Needham (1939), since they did not use the opposite side of the gastrula for comparison with the dorsal lip. My results agree with theirs to the extent that the presumptive epidermis and the dorsal lip consume oxygen at about the same rate when uncorrected for yolk content. The corrected values cannot be calculated until a more precise analysis of the yolk content is made.

With regard to the low rate of oxygen consumption of the yolk-laden cells, Fischer and Hartwig (1938) report that the animal half consumed 3.1 times the O_2 of the vegetal half. The difference observed in this paper shows that there may be a tenfold difference, but the explanation for this greater difference is found in the size of the tissues used for meas-

urement—I used small samples, while they used half of the egg. Further, *A. punctatum* has more yolk than *A. mexicanum*.

SUMMARY

The rate of oxygen consumption of parts of the early gastrula of *A. punctatum* (*A. maculatum*), *A. opacum*, and *R. pipiens* has been measured by a micro Winkler's method, using 300 mm.^{3} of solution.

The results show a gradient in the rate of oxygen consumption which runs from 28 in the region of the animal pole to 4 in the region of the vegetal pole of the gastrula of *R. pipiens*.

A first approximation of the yolk content of various regions shows a gradient running from 20 per cent at the animal pole to 50 per cent at the vegetal pole.

The gradient in rate of oxygen consumption, therefore, when "corrected" for yolk content is from 35 at the animal pole to 8 at the vegetal pole. It must be emphasized that, since we are dealing with gradients, the values above are only approximations and the gradient may become steeper as the samples used for analysis become smaller.

The dorsal lip of the blastopore consumes oxygen at a higher rate than the opposite side of the gastrula. The magnitude of the difference in rate depends on the size of the samples of tissue used.

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PLATE I

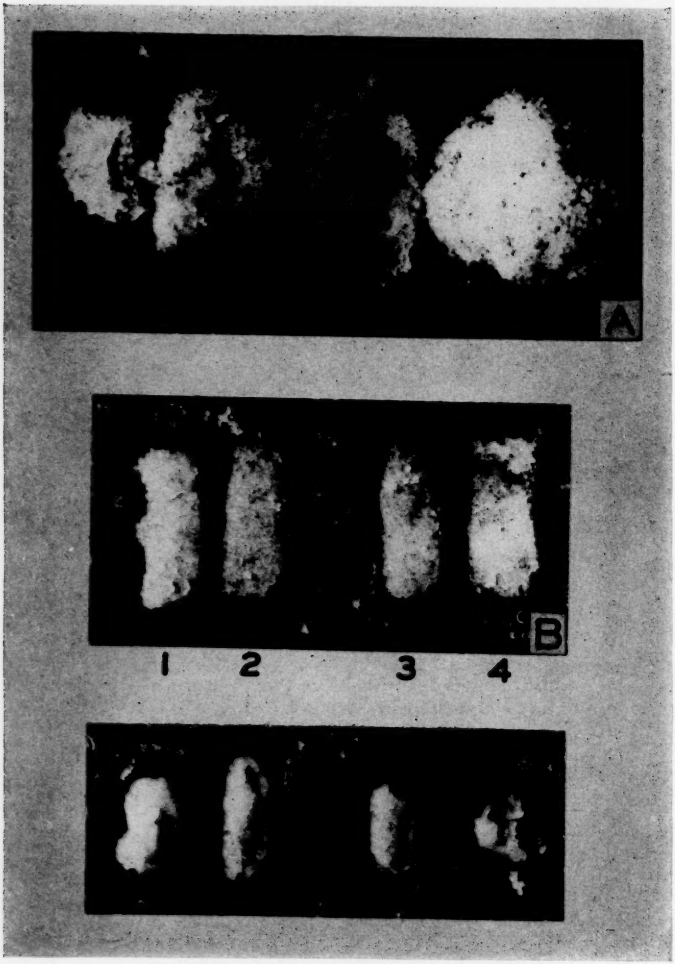

PLATE I

Photographs of a living gastrula of *Amblystoma punctatum*. *A*, Strip of tissue from the ventral to dorsal lip separated from yolk mass and flattened. *B*, Tissue trimmed and cut into four parts: *1*, dorsal lip; *2*, presumptive neural plate; *3*, presumptive epidermis; *4*, ventral lip. Mass of yolk is No. 5 in Fig. 2. *C*, Pieces healing.

THE EFFECT OF DIFFERENT RELATIVE HUMIDITIES, DURING THE PUPAL STAGE, ON THE REPRODUCTIVE CAPACITY OF THE LUNA MOTH, TROPAEA LUNA L.

(Three figures)

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AMONG moths of the family Saturniidae, larvae of certain species (e.g., *Tropaea luna* and *Automeris io*) form cocoons on the ground, where they remain in a moist environment throughout the winter; whereas those of other species (e.g., *Callosamia promethea* and *Samia walkeri*) suspend their cocoons from the branches of trees, where they are subjected to the drying effects of the atmosphere. This observation suggests differences in the humidity requirements of these insects during pupal development. Since the cocoons of some species pass the winter in a moist environment and those of other species in a relatively dry environment, the question arises as to whether there are differences in the ability of the cocoons of these two groups to prevent the evaporation of water from the developing pupae. The present experiments were originally planned to determine the humidity range in which pupal development of the Luna moth, *T. luna*, can occur and also to determine whether the cocoon serves to retain water when pupae are subjected to low humidities. For this purpose, pupae in their cocoons and naked pupae were subjected to constant relative humidities ranging from 0 to 100 per cent and their rates of weight loss determined. Later it is planned to perform a comparable series of experiments with pupae of either *C. promethea* or *S. walkeri*.

The original experiment provided material which made it possible to extend the study to include the effects of development at different humidities on subsequent reproductive processes. The effects of different humidities on oviposition in insects are not uniform. In general, it has been found that high humidities favor oviposition and that the lower the humidity, the fewer the number of eggs deposited (Headlee, 1917; Powell, 1931; Klein, 1932; Sweetman and Wedemeyer, 1933; Ahmad, 1936). On the other hand, Zwölfer (1931) found that in the moth, *Panolis flammea*, more eggs were laid at humidities of 80–90 per cent than at 100 per cent, and Sweetman (1938) observed that 84 per cent was the optimum for oviposition in the firebrat, *Thermobia domestica*. These results were obtained by subjecting insects to different humidities and noting the number of eggs deposited.

A literature review failed to show any experiments in which growing or metamorphosing stages of an insect had been subjected to different humidities and the resulting effects on oviposition recorded. In the present experiments adults were obtained from pupae kept at humidities of 0–100 per cent. Accordingly, it was possible to determine the effects of pupal development at the different humidities on the oviposition of the adults obtained from these pupae. Members of the family Saturniidae are particularly suitable for this type of experiment because the adults take no food or water which might influence the number of eggs produced. Since fertile eggs were obtained from moths which

had developed at different humidities, the study was extended to include the effects of humidity on the survival of these eggs and on the weight and water content of the resulting larvae.

MATERIAL AND METHODS

Luna cocoons were obtained during the fall of 1939 from a biological supply company and immediately placed under out-of-door conditions, where they were kept until needed for experimentation. Under these conditions the low temperature during the winter months inhibits pupal development. During the spring months development of the pupae not previously used in these experiments was inhibited by artificial refrigeration. In all experiments the pupae were divided into two series. In the first series the pupae within their cocoons were placed separately in glass vials, weighed on a chainomatic balance sensitive to 0.1 mg., and then transferred to humidifiers at the desired humidity. In the second series the cocoons were cut lengthwise and the pupae removed. They were then placed in glass vials, weighed, and also transferred to humidifiers. In each series twenty pupae were used at each humidity studied. Pupal-weight readings were made daily until the emergence of the moth. The approximate time of emergence from naked pupae could be determined in advance by noting the coloring of the wings and the brittleness of the pupal covering. The time at which emergence was expected from pupae within their cocoons was determined by cutting a small slit in each cocoon and making daily observations on wing coloration. No differences were observed in the rates of weight loss at any humidity as a result of opening the cocoon in this manner. Usually the opening was not made until a few days before emergence. In both series, even in saturated air, pupae lost weight more rapidly during the days just preceding emergence. Hence, after several experiments, the approximate time of emergence in the first series could be estimated, particularly at low humidities, by observing the rate of weight loss. When the moth was ready to emerge, the vial containing the pupa was removed from the humidifier and placed in a screen cage in the laboratory.

The empty cocoons were also placed in glass vials, weighed, and exposed to different humidities. They were weighed daily until constant weights were obtained.

After emergence, male and female moths were paired in screen cages and kept under laboratory conditions. Approximately one-fourth of the pairs were observed to mate. Copulation usually began in the evening before 11:00 P.M., and the pairs remained attached until the following afternoon or evening. No correlation was observed between the humidity to which the pupae had been exposed and the success in obtaining matings.

The eggs from both mated and unmated females were collected daily. The infertile eggs were used for the determination of water content, and the fertile eggs for a study of the effect of different humidities on the survival, on the rate of embryonic development, and on the weight and water content of the resulting larvae. For the determination of water content, the eggs were weighed, and then dehydrated to constant weights at approximately 60° C. Water percentages were calculated from the weight readings obtained before and after complete desiccation. The fertile eggs were placed in small homeopathic vials plugged with loosely packed, nonabsorbent cotton to prevent the escape of the newly hatched larvae and to permit the free passage of air into and out of the vials. They were then weighed and placed in humidifiers at the desired humidity. Weight readings were made daily until the larvae hatched. During the hatching period the larvae were removed from the vials each day; and, whenever ten or more had hatched at a particular

humidity, they were weighed and desiccated to a constant weight at approximately 60° C. for the determination of water content.

Constant relative humidities were obtained by the use of saturated solutions of various salts in the bases of ordinary desiccators. The approximate relative humidity values of these solutions were obtained from the data given by Chapman (1931, p. 95) and by Sweetman (1933). A saturated atmosphere was obtained with distilled water and a dry

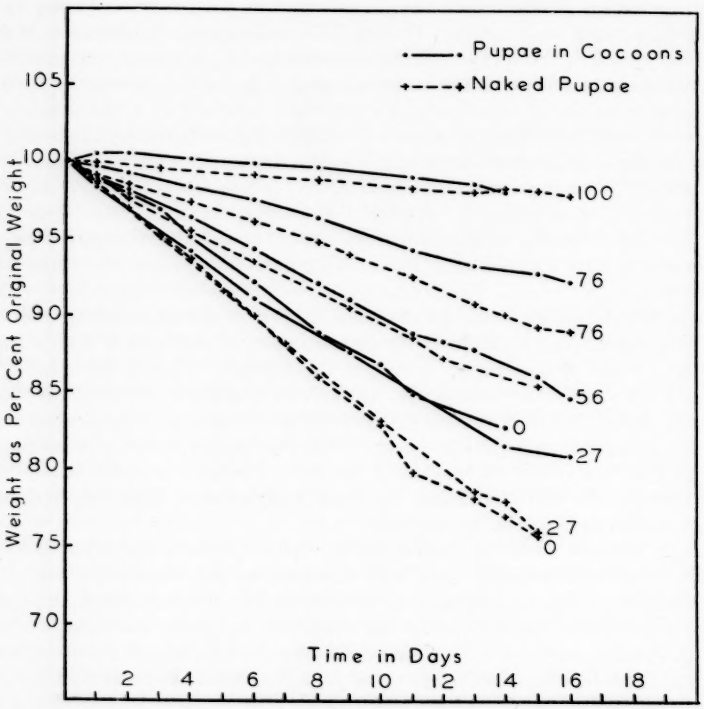

FIG. 1.—Rate of weight loss of *T. luna* pupae at different relative humidities. Numbers indicate relative humidities.

atmosphere by the use of either anhydrous $CaCl_2$ or anhydrous magnesium perchlorate. Throughout the period of experimentation the humidifiers containing the pupae and eggs were kept in an incubator at a temperature of approximately 25° C.

OBSERVATIONS

The rates at which weight was lost by pupae when taken from out-of-door conditions and placed at different humidities are plotted in Figure 1. Each curve in this figure represents the average weight loss of twenty individuals. An examination of the figure shows that pupae lost weight at all humidities, including 100 per cent. Pupae left in

their cocoons and placed in saturated air appear to have gained weight during the first four days of exposure. This increase in weight was due to the imbibition of water by the cocoon and not by the pupa (see Fig. 2). Although the curves appear regular, there was considerable variation in the rates at which different individuals lost weight under identical conditions. Much of this variation was due to the fact that some of the pupae began to develop immediately on being placed at 25° C., whereas others, as evidenced by their later emergence, remained dormant for different periods of time even at this relatively high temperature. Dormant pupae were found to lose weight at a slower rate than developing pupae. This observation suggests that the rate of weight loss, under the condi-

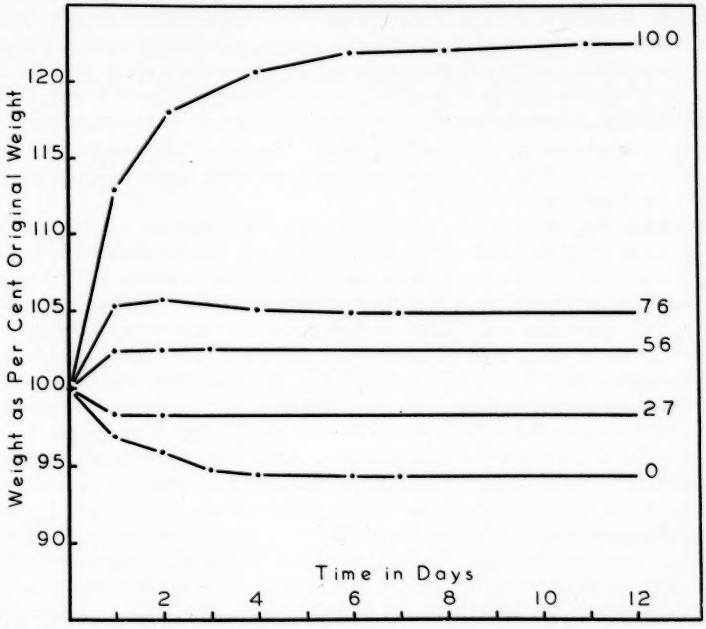

FIG. 2.—Changes in weight of the empty cocoons of the Luna moth at different relative humidities. Numbers indicate relative humidities.

tions of this experiment, is determined by two factors operating simultaneously, namely, (1) the oxidation of stored foods by the pupa and (2) dehydration. In dormant pupae metabolism, with the resulting loss of tissue solids, occurs at a slower rate than in actively developing pupae. The loss of tissue solids is presumably the same at all humidities for the pupal stage of an insect, whereas the loss of weight by dehydration is greater at lower humidities (Evans, 1935).

Figure 1 shows that pupae subjected to different humidities while in their cocoons lost weight at a slower rate than naked pupae. This difference is more pronounced at 0 per cent humidity. However, at this humidity many of the pupae left in their cocoons remained dormant for several weeks of the experiment; four of them required more than

one month to complete development. With the exception of this group, the lower the relative humidity, the greater the rate of weight loss. Many pupae also remained dormant for several weeks at 100 per cent humidity. However, the slopes of the curves were not modified at this humidity, since weight loss, representing only the loss of tissue solids, occurred very slowly. Corresponding to the more rapid loss of weight, pupae placed at low humidities lost more weight before the completion of development than those placed at high humidities. Thus when pupae were placed at 100 per cent, the moths emerged when the pupal weights were approximately 95 per cent of the original values, while those at 0 per cent lost nearly 30 per cent of their original weight before emergence.

The changes in the weights of empty cocoons at different humidities are plotted in Figure 2. The cocoons were found to lose weight at 0 and 27 per cent and to gain weight at 56, 76, and 100 per cent. A rapid and prolonged gain in weight occurred in saturated air. Here the cocoons continued imbibing water over a period of more than ten days, and their weights at equilibrium were more than 120 per cent of the original values; whereas, at other humidities, constant weights were attained after two to four days' exposure, and the changes in weight were comparatively small. The imbibition and retention of water by the Luna cocoon in moist air undoubtedly serves to keep the pupa at a high humidity throughout its development.

Table 1 shows that most of the pupae completed development at each humidity, although there was a slightly higher mortality at the lower values; the percentage of moths emerging varied from 85 at 0 per cent to 100 at 76 per cent and 100 per cent humidity. The time required for the pupae to complete development was longer at high than at low humidities, with the exception of the one group at 0 per cent referred to above. However, in each group, even at the high humidities, there were a few individuals which completed their development in two weeks or less. For this reason and also because the entire pupal period is not considered but only the time from winter dormancy to adult emergence, the differences in the time required for the completion of the pupal stage at different humidities are not considered significant. Since nothing is known concerning the previous history of the individuals studied, it is difficult properly to evaluate the time figures given in the table. However, in view of certain observations made in the laboratory on nondormant pupae, it seems likely that very little development occurs in overwintering pupae before dormancy begins. Six Luna pupae were obtained during July, 1940, from larvae reared in the laboratory and kept under observation until adult emergence. The average duration of the pupal stage in these individuals, calculated from the time of spinning until adult emergence, was only eighteen days. This figure compares favorably with those given in Table 1 for the completion of the pupal stage following winter dormancy.

The average numbers of eggs laid by moths which completed pupal development at different humidities are listed in Table 1. Subjecting the pupae to humidities between 56 and 100 per cent did not seem to affect the average number of eggs laid per female. However, below 56 per cent, the number of eggs deposited appears to depend on the amount of water lost during pupal development. Averaging the number of eggs laid by moths of both series which completed development at a particular humidity gave only 85 at 27 per cent and only 51 at 0 per cent, as compared with 184 at 100 per cent humidity. At 56 per cent, females which developed from pupae left in their cocoons lost only 15 per cent of their original weight before emergence and laid an average of 182.2 eggs, while females which developed from naked pupae lost 17.4 per cent of their original weight and laid

only 123.3 eggs each. Hence, a weight loss of 15 per cent occurred before any effect on oviposition was noted. A loss of from 15 to 17.4 per cent of the original weight of the pupa apparently includes a critical value at which dehydration first shows its effect on number of eggs produced.

The figures on oviposition listed in Table 1 include observations on both mated and unmated females. Table 2 shows that mating did not influence the average number of

TABLE 1

EFFECT OF DIFFERENT RELATIVE HUMIDITIES ON THE PUPAE OF THE LUNA MOTH
(Numbers in parentheses indicate the numbers of eggs weighed or dehydrated)

Approximate Humidity (Per Cent)	Per Cent Emerged	Time Required To Complete Development	Weight Just before Emergence, Per Cent Original Weight	No. of Eggs per Female (Average)	Weight of Eggs in Milligrams (Average)	Average Water Content of Eggs (Per Cent)
100 (pupae in cocoons)...	100.0	27.8±2.09	94.4	181.1	2.67 (3,030)	67.4 (2,482)
100 (naked pupae).....	95.0	23.0±1.47	95.4	186.7		
76 (pupae in cocoons)....	100.0	22.4±1.48	88.4	202.5	2.66 (2,157)	67.2 (1,110)
76 (naked pupae).....	95.0	19.3±1.17	86.6	171.7		
56 (pupae in cocoons)....	95.0	16.1±0.48	85.2	182.2	2.65 (2,388)	67.8 (1,439)
56 (naked pupae).....	95.0	17.5±0.56	82.6	123.3		
27* (pupae in cocoons)...	90.0	18.1±1.17	74.4	82.8	2.31 (1,607)	67.0 (1,607)
27 (naked pupae).....	90.0	17.3±0.77	72.8	88.2		
0 (pupae in cocoons).....	85.0	23.3±2.47	72.8	60.6	2.23 (374)	66.6 (270)
0 (naked pupae).....	90.0	16.8±0.86	70.6	41.9		

*This humidity was obtained with $KC_2H_3O_2$. Sweetman (1933*b*) gave 25 per cent as its humidity at 22°C. and 29 per cent at 27°C. Since the experiments given above were performed at 25°, the humidity value is given as 27 per cent. Janisch (1933) gave 19.0 per cent as the humidity for this salt at 25°.

eggs laid by each female. However, unmated females required more than eight days to complete oviposition, while fertilized females laid their eggs in approximately six days.

The data on the weight of eggs laid by moths which developed at different humidities, listed in Table 1, include weight determinations on both fertile and infertile eggs. Since no differences could be noted between their weights, it was assumed that their water contents were also the same. No differences in either the weights or the percentages of water were noted in eggs laid by females which had completed development at humidities from 100 to 56 per cent. The weight of eggs laid by females which developed at 56 per cent or above averaged 2.66 mg.; while moths which developed at 27 per cent laid eggs which

weighed only 2.31 mg., and the eggs of those exposed to 0 per cent weighed 2.23 mg. Corresponding to these differences in weight, there was a slightly reduced water content in eggs laid by moths which had developed at the lower humidities.

Table 3 contains the observations made on fertile eggs of moths which developed under different humidity conditions. Complete data were obtained only on the eggs of moths emerging at humidities of from 56 to 100 per cent. For the lower values only a few fertile eggs were obtained. The data show no differences in the mortality of eggs laid by moths which developed at 76 or 100 per cent humidity. However, those which developed at 56 per cent laid eggs which showed a higher mortality at the lower humidities. This result is difficult to explain in view of the observation that these eggs had as high a water content as those laid by moths which developed at higher humidities. However, it must be noted

TABLE 2

EFFECT OF MATING ON THE NUMBER OF EGGS LAID BY THE LUNA MOTH AND ON THE DURATION OF THE OVIPOSITION PERIOD

Approximate Humidity at Which Female Emerged (Per Cent)	Mated Females			Unmated Females		
	No. of Females	No. of Eggs per Female	Duration of Oviposition (Days)	No. of Females	No. of Eggs per Female	Duration of Oviposition (Days)
100	3	198.3	6.3	17	183.5	8.8
76	5	192.8	5.6	11	181.7	8.0
56	4	169.3	7.0	11	171.9	8.6

that observations on water content could not be made on the eggs used in this experiment but were made only on infertile eggs. In each experiment the optimum humidity for embryonic development was 76 per cent. However, since humidities between 76 and 100 per cent were not employed, the optimum humidity may be higher than indicated. A high mortality occurred in saturated air, probably because in many cases mold growths appeared on the eggs. Even at the optimum humidity, whenever a considerable number of eggs were used, not more than 80 per cent of them hatched. Another feature of considerable interest is the high percentage of survivals obtained at 0 per cent humidity, where 26.3 per cent of the eggs laid by moths which had developed at 56 per cent humidity hatched, and 44.2 per cent of those laid by moths which developed in saturated air also hatched. Although very few eggs were available, it was noted that even when a moth developed at a very low humidity, its eggs were able to hatch if allowed to develop at an optimum humidity. However, in these cases mortality was very high at less favorable humidities.

The data contained in Table 3 indicate that at 25° C. humidity has no effect on the duration of the embryonic development of the Luna moth. Eggs which are exposed to dry air will hatch just as quickly as those placed at more favorable humidities. It was also observed that the duration of the hatching period was not influenced by humidity, the eggs hatching from the seventh to the eleventh days in saturated air and from the eighth to the eleventh days in dry air.

Table 3 also lists the weights and the water contents of larvae which hatched under

TABLE 3

THE EFFECT OF RELATIVE HUMIDITY ON THE HATCHING OF THE EGGS OF THE LUNA MOTH AND ON THE WEIGHT AND WATER CONTENT OF THE LARVAE

Approximate Humidity (Per Cent)	Number of Eggs	Per Cent Hatched	Time in Days	Larvae	
				Weight in Milligrams	Per Cent Water
	Females Emerged at 100 Per Cent R.H.				
100	160	41.9	9.29	2.43	79.2
76	162	74.7	9.69	1.97	76.8
56	187	49.2	10.49	1.97	73.7
27	175	44.6	9.86	1.55	.....
12	191	67.5	9.45	1.61	72.5
0	163	44.2	9.80	1.58	70.5
	Females Emerged at 76 Per Cent R.H.				
100	168	72.6	9.32	2.24	78.3
76	119	80.7	8.86	1.89	76.0
56	149	65.8	8.72	1.87	75.3
27	131	38.9	9.72	1.58	72.2
12	123	50.4	9.61	1.46	67.9
0	120	45.8	9.96	1.39	68.7
	Females Emerged at 56 Per Cent R.H.				
100	100	61.0	8.90	2.08	78.7
76	108	73.1	9.16	1.83	75.0
56	89	64.0	9.53	1.72	75.8
27	72	48.6	8.57	1.73	73.4
12	50	14.0	9.28	1.50	.....
0	95	26.3	9.20	1.42	.....
	Females Emerged at 27 Per Cent R.H.				
76	60	16.6	9.40	1.72	.....
56	50	4.0	10.00	.....	.....
	Females Emerged at 0 Per Cent R.H.				
100	78	0.0	.....	.....	.....
76	26	92.3	10.3	1.62	77.4

different humidity conditions. In each group larval weight decreased with the relative humidity to which the eggs were exposed. In some instances the larvae which hatched from eggs laid by females which developed at low humidities did not weigh as much as others hatched at the same humidities but from eggs laid by moths which developed at higher humidities. However, weights of larvae of different groups which hatched at the same humidities are not consistently different. Corresponding to the decrease in weight at low humidities, the water content of larvae was also less than that of larvae which hatched at high humidities.

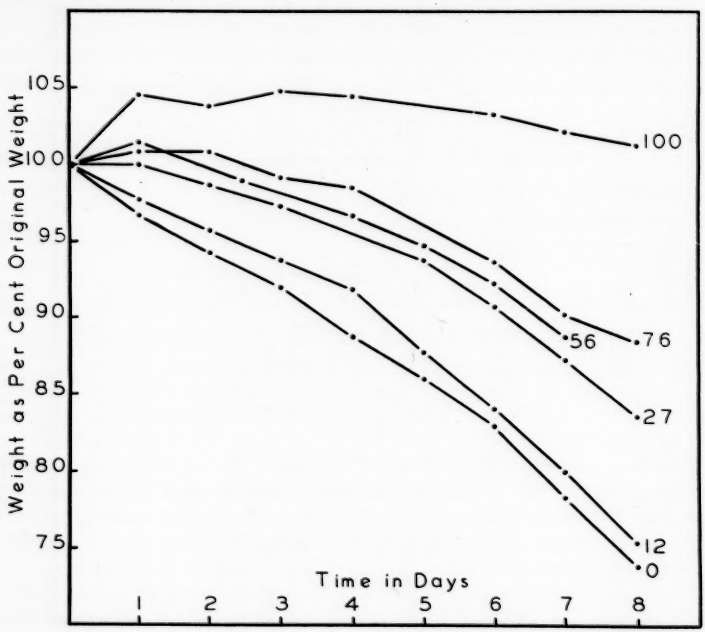

FIG. 3.—Rate of weight loss of *T. luna* eggs at different relative humidities. These eggs were laid by moths which had developed at 76 per cent humidity. Numbers indicate relative humidities.

Figure 3 contains graphs showing the changes in weight of eggs exposed to different relative humidities. Those placed in saturated air imbibed water during the first day of exposure and maintained a high water content throughout embryonic development. At humidities of 76 and 56 per cent there was a slight increase in weight during the first day of exposure, but this was followed by a steady loss of weight until, at the time of hatching, the eggs weighed less than 90 per cent of their original weight. At low humidities there was a steady weight loss throughout embryonic development, and at 0 per cent the eggs had lost more than 25 per cent of their original weight when the larvae hatched. In spite of this weight loss, approximately 45 per cent of the larvae hatched (Table 3). Hence the imbibition of water by the egg of the Luna moth is not necessary for normal embryonic development.

DISCUSSION

Many insects are hygroscopic and can absorb water from air which has a high moisture content. This property has been demonstrated for the adult potato beetle, *Leptinotarsa decemlineata*, by Breitenbecher (1918); for the larva of *Tenebrio molitor* by Buxton (1930); for the hibernating nymphs of the grasshopper, *Chortophaga viridifasciata*, by Bodine (1921); and for the growing nymphs and the adults of the same species by Ludwig (1937). On the other hand, the results just described show that the pupa of the Luna moth, *T. luna*, loses weight at all humidities, including saturated air. Similar observations were made by Evans (1935) for the prepupae and pupae of the blow-fly, *Lucilia sericata;* by Ludwig and Landsman (1937) for the larvae, prepupae, and pupae of the Japanese beetle, *Popillia japonica;* and by Theodor (1936) for the pupae of the fly, *Phlebotomus papatasii*. However, in the present paper it has been shown that the cocoon of the Luna moth is hygroscopic and imbibes a large amount of water when placed in saturated air. Thus it serves to absorb and retain moisture for the developing pupa.

Although the Luna cocoon is made on the ground and in this location is always kept moist, the results of the present study indicate that an environment containing a high moisture content is not essential for normal pupal development, adults emerging even in dry air. Since these experiments included only the part of pupal development from winter dormancy until adult emergence, it is possible that the entire pupal development would require a higher humidity than is indicated by the observations. Theodor (1936) observed that the pupae of *P. papatasii* lost water at 30° C. and humidities of 80–100 per cent. He found that complete development was not possible under these conditions, the pupae dying in a late stage of development, sometimes shortly before emergence. However, adults were obtained when pupae were placed at 30° C. and at humidities of 80–100 per cent in the middle of their development because, according to Theodor, development was completed before the fatal limit of desiccation was reached. It is also possible that the pupae in the middle of their development were in a more resistant condition than was the case at the beginning of the pupal stage and consequently were able to survive adverse environmental conditions.

Since in the Luna moth oviposition may begin from twenty-four to forty-eight hours after emergence, the eggs ripening during the pupal stage, humidity experiments on the pupae may be comparable to similar experiments on the adults of many other insects. Furthermore, the moth takes no food or water which might influence oviposition. It was observed that subjecting Luna pupae to humidities ranging from 56 to 100 per cent had little or no effect on the average number of eggs laid by each female obtained from these pupae, or on the weight and water content of the eggs. This range of optimum humidities is greater than that so far reported for the oviposition of other insects when subjected to different relative humidities. Headlee (1917) obtained an average of 43 eggs per female when the bean weevil, *Bruchus obtectus*, was subjected to 80° F. and 100 per cent humidity, but only 26 eggs per female at the same temperature and 62 per cent humidity. He observed no further reduction in the number of eggs at lower humidities. Zwölfer (1931) observed that in the moth, *Panolis flammea*, the number of eggs produced was greatest at humidities between 80 and 90 per cent, at temperatures between 8° and 27° C. At 100 per cent there were very few eggs laid. Sweetman (1938) found in his study of the firebrat, *Thermobia domestica*, that the optimum humidity for oviposition was 84 per cent at favorable temperatures and that oviposition was possible only at humidities between 48 and 97 per cent.

Unfavorable humidities may affect the embryonic development of an insect by modifying its rate and by increasing mortality. The developmental rate of the Luna eggs was not influenced by humidity at a temperature of 25° C.; the larvae hatched at the same time in a saturated atmosphere and in dry air. Similar observations were made by Holdaway (1932), using the egg of the flour beetle, *Tribolium confusum;* by Maercks (1933) on the egg of the wasp, *Habrobracon juglandis;* by Sweetman (1938) with the egg of the firebrat, *T. domestica;* and by Geisthardt (1937) and Johnson (1940) on the eggs of the bedbug, *Cimex lectularius*. However, in many cases a slower developmental rate has been observed at low humidities (Andersen, 1930; Janisch, 1930; Wardle, 1930; Powell, 1931; Pyenson and Sweetman, 1931; Evans, 1934; and Steiner, 1937). Clark (1935) found that with the egg of the bug, *Rhodnius prolixus*, the only difference in rate of development due to humidity was a lag in saturated air. In the majority of instances, the above workers found that exposing insect eggs to saturated air increased mortality because of the growth of fungus on the eggs. It was observed that mortality of the Luna egg was higher in saturated air and that in many cases the eggs were covered with a fungal growth. A higher mortality of Luna eggs also occurred at low humidities. This result is in agreement with the observations of most investigators. However, Sweetman (1938) with the egg of the firebrat, *T. domestica*, and Johnson (1940) with the egg of the bedbug, *C. lectularius*, found that humidity had little effect on survival over the optimum temperature range. Holdaway (1932) observed that the viability of the egg of *T. confusum* was highest in dry air.

The swelling of the egg due to the imbibition of water is a necessary accompaniment of development in many insects. This was demonstrated for the grasshopper, *Melanoplus differentialis* by Bodine (1929); for the beetle, *Anisoplia austriaca* by Kerenski (1930); for the Japanese beetle, *P. japonica* by Ludwig (1932); and for the bug, *Notostria erratica* by Johnson (1937). Needham (1931, p. 905) states that "swelling in fact seems to be a regular occurrence in the development of the eggs of all Tenthredinidae, Cynipidae, and Formicidae." In many cases the egg more than doubles its weight during the first few days of development. Figure 3 shows that the egg of the Luna moth also imbibes water when placed in a moist atmosphere. However, this imbibition of water is not essential to the normal development of the embryo as evidenced by the high percentages of hatching which were obtained at low humidities where absorption of water was not possible.

The Luna moth appears to be very well adapted for survival under adverse humidity conditions. Its cocoon is made between leaves on the ground where conditions are normally very moist. It has been shown (Fig. 2) that under such conditions the cocoon has the ability to absorb a large quantity of water. Should the humidity of the environment become low, the cocoon would retard the evaporation of water from the pupa and in this way would help it to survive. It has been found (Table 1) that moths can emerge even in dry air and that such adults mate and lay eggs which hatch under favorable humidity conditions (Table 3). Adults which emerge at more favorable humidities (56–100 per cent) lay eggs, a large percentage of which hatch at all humidities, including dry air.

SUMMARY

1. Luna pupae were found to lose weight at all humidities, including saturated air. In general the lower the humidity, the greater the rate of weight loss and the greater the total weight loss before adult emergence. Thus when pupae were placed in saturated air

the moths emerged when the pupal weights were approximately 95 per cent of the original values, while those in dry air lost nearly 30 per cent of their original weight before emergence.

2. The cocoon of the Luna moth is hygroscopic, absorbing a large amount of water when placed in a moist environment. Pupae within their cocoons did not lose weight as rapidly as naked pupae. The cocoon probably serves to keep the pupa in a moist environment throughout its development.

3. Subjecting pupae to humidities of between 56 and 100 per cent had no effect on subsequent oviposition or on the weight and water content of the eggs laid. However, when pupae were subjected to lower humidities, there was a reduction in the number of eggs laid per female and also in their weight and water content.

4. Eggs laid by moths which had emerged at humidities ranging from 56 to 100 per cent were subjected to different relative humidities, and their time of development, mortality, and the weight and water content of the resulting larvae were determined. From this study the following conclusions were derived:

a) At a temperature of 25° C. humidity had no effect on the duration of the embryonic period.

b) The optimum humidity for embryonic development was approximately 76 per cent. A high mortality occurred in saturated air, probably due to the growth of molds on the eggs. At humidities below 76 per cent, the number of survivors was also reduced, although a high percentage of the eggs hatched even in dry air.

c) Larval weight and water content were found to decrease with the humidity at which embryonic development occurred.

5. Moths which had emerged at low humidities (0 and 27 per cent) laid eggs, a high percentage of which hatched at optimum humidities. However, the mortality of these eggs was very high under less favorable conditions.

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THE ACTION OF DRUGS ON THE COMPOUND ASCIDIAN, PEROPHORA VIRIDIS, AS INDICATED BY THE ACTIVITY OF THE INTACT HEART

(Five figures)

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THE ascidian heart is without parallel among the Chordata. It is said to lack valves and blood vessels; and, while it reverses the direction of beat rhythmically and consistently, the number of beats in one direction is usually larger than in the other. Furthermore, the counts in both directions are highly variable even in the quiescent animal. At present some difference of opinion exists as to the presence or absence of a nerve supply to this organ or within the organ itself. The heart, in some ascidians at least, is quite resistant to experimental manipulation and may be removed from the body or even cut into pieces without inhibiting its activity.

Some study has been made of the response of the ascidian heart to various drugs and vertebrate hormones with somewhat conflicting, or at most scanty and negative, results. Although acetylcholine has not been demonstrated in ascidian tissues (Bacq, 1935*b*), choline esterase (Bacq, 1935*c*) has been found. According to Bacq (1935*a*), weak concentrations of acetylcholine and adrenalin have no significant effect on the frequency of the isolated heart of *Ciona intestinalis*, while strong concentrations can cause an abrupt arrest of automatic action. Dilution causes normal activity to reappear almost at once. After an arrest provoked by acetylcholine, heart rhythm is more rapid, while after adrenalin the frequency is less than normal. This result may point to a difference in the action of the two drugs. Adrenalin is said to accelerate beating in the intact heart of *Salpa* (Hykès, 1926). Hunter (1903) has studied the action of adrenalin and strychnine on the heart of *Molgula* but mentions no results for adrenalin other than that general effects conform with experiments on vertebrates.

The present paper is based on the results obtained from exposure of the compound ascidian, *Perophora viridis*, to acetylcholine, mecholyl, adrenalin, atropine, strychnine, and colchicine.

MATERIALS AND METHODS

In *P. viridis* the small, transparent, greenish-colored zoöids are connected by stolons in which circulation of the blood occurs. Certain observations seem to indicate a possible co-ordination of the heart beat between individuals on the same piece of stolon. The hearts of these different individuals may not reverse at the same moment, but for the most part all within the microscopic field were in the same phase. After isolation this apparent co-ordination is lost. Isolation by cutting the stolon has no appreciable effect on the activity of the heart. The heart beat consists of a wavelike pulsation which starts at one end of the tube and carries through to the other. Unlike certain other

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ascidians which have been reported in the literature, the number of beats in the abvisceral phase (toward the endostyle and stolon) generally exceeds the advisceral. In spite of the constant variation in the number in both phases (Table 1), this difference is usually maintained. The length of the interval between reversals seldom exceeds 1 second except under certain experimental conditions.

Stock solutions of acetylcholine chloride, mecholyl (acetyl-beta-methylcholine-chloride), adrenalin chloride, atropine sulphate, strychnine sulphate, and colchicine were made at daily intervals and appropriate dilutions made previous to their use. Before the animals were transferred to the experimental solutions, the condition and rate of the normal, quiescent heart were determined, as well as the length of the rest periods and the limit of variability in the number of beats in both directions. These served as a control for the action of the drugs, together with counts made when the animals were transferred from one dish of sea water to another. Since the number of beats in either phase varies in the same and in different animals, a consecutive series of counts was made, from which the highest and lowest for specific cases are included in Tables 1 and 2. All experiments were made at room temperature, which varied from day to day between 18° and 24° C. Indicators of the effects produced upon the heart function included change in number of beats in both phases, amplitude and quality of beats, length of the two phases as measured with a stop watch, length of interval between reversals, and recovery in experimental solution and sea water. The vigor of the contractions could only be estimated.

EXPERIMENTAL

Acetylcholine.—This drug usually increased the length of most abvisceral phase counts for a short time following the beginning of exposure, but for the most part had only a depressive action on the advisceral (Fig. 1). In strong concentrations (about 1:1,000 to 1:5,000) the dominance of the abvisceral phase gradually ceased, the number of beats in either phase became highly variable, and the heart eventually stopped beating without undergoing reversal. Before activity entirely ceased, the beating was reduced to pulsations at both ends, impulses traveling only part way along the tube. With continued exposure, beating was again resumed in an irregular fashion at first as a result of the destruction of the drug. A brief summary of the effects produced on heart activity in some of the experimental animals is given in Table 1.

Figure 1 (Case D, Table 1) is illustrative of the abrupt and variable increase in the number of beats in successive abvisceral phases. In this instance the period extended for about 25 minutes before a return to the normal condition previously determined on the fresh animal. The advisceral phase showed no significant change in numbers of beats between reversals. Not all animals reacted in this manner, e.g., in Cases J and H the counts in both phases were slightly depressed. Regardless of size, little appreciable change was noted in the rate of heart beat during either phase. Exceptions occurred toward the end or beginning of either phase and occasionally during a lengthy abvisceral phase, when the rate was noticeably slower for several seconds, only to be followed by typical activity in the same phase. This latter type of behavior seems to point to a temporary weakening of the abvisceral dominance, but not to such an extent that reversal usually occurred. If reversal ensued, the length of the advisceral phase was reduced to one or several beats at most (Fig. 2). Change in amplitude or strength of the beat was noticeable only at strong concentrations or at times of marked inhibition.

TABLE 1

EFFECT OF ACETYLCHOLINE ON THE ACTION OF THE HEART OF *Perophora viridis*

CASE AND CONCENTRATION	HIGH AND LOW COUNTS OF NORMAL				MINUTES STUDIED	HIGH AND LOW COUNTS OF EXPERIMENTAL				SIZE OF ANIMAL	ROOM TEMPERATURE (° C.)	COMMENTS
	Abvisceral Phase		Advisceral Phase			Abvisceral Phase		Advisceral Phase				
	Number of Beats	Time in Minutes	Number of Beats	Time in Minutes		Number of Beats	Time in Minutes	Number of Beats	Time in Minutes			
B	182	2:36	145	2:07	225	Stopped beating 5 minutes after exposure was begun				Small	21	Irregular beating in both phases later about the same. Variation in counts 3–103
1:4,000	142	2:00	131	1:56								
A	166	2:40	133	2:08	45	257	4:01	4	.....	Medium	22	Advisceral counts immediately reduced to 1–3. A few abvisceral counts increased but otherwise depression. Two phases later equal
1:25,000	144	2:10	97	1:35		24	0:19	1	.....			
C	133	2:07	117	1:54	52	136	1:55	130	1:52	Medium	23	Initial irregular depression in counts in both phases, followed by recovery in time
1:50,000	109	1:51	108	1:37		8	.....	7	.....			
D	120	1:54	102	1:32	61	224	3:15	102	1:35	Large	20	First few abvisceral counts markedly increased, with slight depression in advisceral
1:100,000	112	1:47	85	1:18		111	1:35	75	1:11			
H	146	2:34	119	2:09	151	131	1:45	107	1:40	Small	21	Much like Case D above. Fewer high counts and longer advisceral depression in number
1:100,000	124	2:10	105	1:45		105	1:25	89	1:22			
E	142	2:17	113	1:59	54	143	2:05	124	2:00	Medium	21	Slight depression at first in both phases, followed by quick recovery and less variation at highest counts
1:125,000	102	1:41	84	1:31		124	1:57	96	1:42			
J	184	3:56	138	2:54	71	164	3:22	135	2:42	Large	19	No effect at first, followed by slight depression lasting for several hours
1:175,000	161	3:22	129	2:47		123	2:35	110	2:16			
K	137	2:33	92	1:53	61	159	2:45	113	2:12	Large	23	Slight increase in abvisceral counts at first. No depression
1:300,000	130	2:30	104	2:09		123	2:12	82	1:45			
A1	143	2:55	89	1:53	44	173	3:02	130	2:24	Small	21	Progressive increase in abvisceral counts at first *plus* depression of advisceral, followed by return to normal numbers
1:300,000	131	2:36	73	1:28		126	2:26	111	2:10			

TABLE 2

EFFECT OF MECHOLYL ON THE ACTION OF THE HEART OF *Perophora viridis*

Case and Concentration	High and Low Counts of Normal				Minutes Studied	High and Low Counts of Experimental				Size of Animal	Room Temperature (° C.)	Comments
	Abvisceral Phase		Advisceral Phase			Abvisceral Phase		Advisceral Phase				
	Number of Beats	Time in Minutes	Number of Beats	Time in Minutes		Number of Beats	Time in Minutes	Number of Beats	Time in Minutes			
K	145	2:16	129	1:57	446	102	2:01	97	1:53	Medium	20	Heart stopped for 15 minutes. Resumed beating with almost equal counts in both phases. At first very irregular and unequal
1:10,000	131	2:02	112	1:45		11	.....	13	.....			
M	149	2:15	135	2:11	185	241	4:46	153	2:40	Medium	22	Marked irregularity *plus* great depression in advisceral phase, followed by recovery. At times marked increase in abvisceral
1:15,000	135	2:05	127	2:05		24	0:33	1	.....			
L	153	2:32	97	1:34	233	229	3:49	130	2:21	Medium	23	Initial great increase in abvisceral counts and depression in advisceral, followed by recovery within several hours
1:20,000	113	1:55	78	1:24		120	2:10	2	.....			
J	134	2:08	111	1:55	460	814	12:47	124	2:22	Medium	21	Great irregularity with a few high counts in abvisceral. Immediate great depression in advisceral. Slow recovery during 8 hours
1:25,000	123	1:55	94	1:28		17	0:21	1	.....			
E	136	2:24	108	1:54	151	352	5:05	73	1:26	Large	19	Increase in some abvisceral counts during first 18 minutes. After 2 hours, counts in both phases depressed and about equal
1:25,000	121	2:03	92	1:26		3	.....	1	.....			
D	116	2:12	132	2:31	245	318	5:47	101	2:10	Medium	22	Initial increase in some abvisceral counts followed by depression. Immediate depression in advisceral. Like Case E above. Recovered by next day
1:50,000	104	1:59	117	2:16		22	0:26	1	.....			
C	133	2:21	114	1:59	206	303	4:58	140	2:36	Small	20	First two abvisceral counts increased, then gradual depression. Initial depression in advisceral, then returned to normal
1:50,000	122	2:12	96	1:38		24	0:22	2	.....			
B	145	2:57	123	2:45	338	195	4:31	127	2:30	Medium	20	Initial increase followed by decrease in abvisceral counts. Great decrease in advisceral. Return to normal by 1½ hours
1:100,000	127	2:15	102	2:13		30	0:44	1	.....			
A	178	3:57	131	3:17	212	175	3:15	126	2:28	Very large	18.5	Increased irregularity in both phases, with depression 4 hours later
1:300,000	133	3:04	115	2:57		13	.....	3	.....			

Figure 2 (Case A, Table 1) shows an initial period of exaggerated abvisceral variation, including some high counts for certain phases, which lasted approximately 15 minutes; a second period involving depression in the number of beats below the level for the fresh

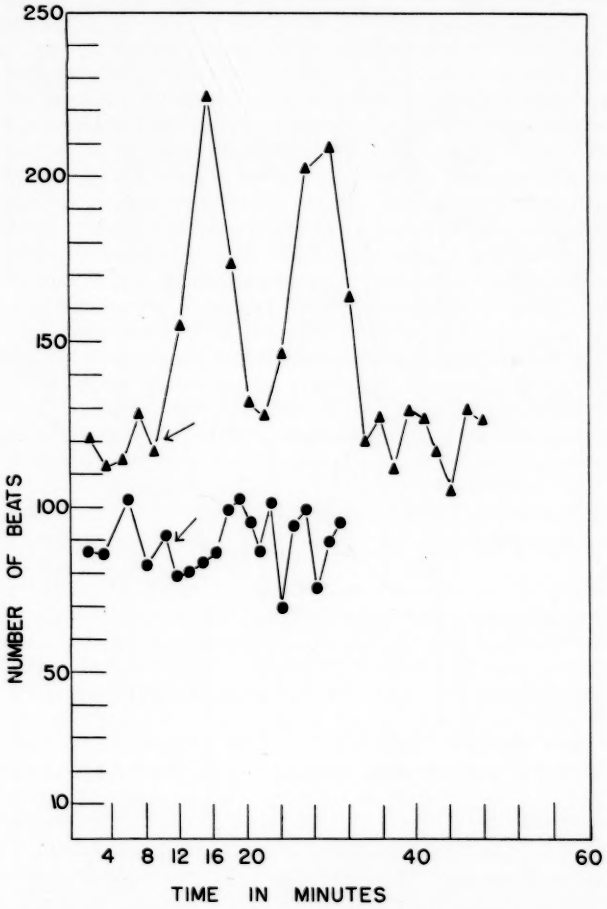

FIG. 1.—Variation in the number of heart beats in consecutive abvisceral phases (triangles) and in consecutive advisceral phases (circles) before and after exposure of a single intact animal to acetylcholine (1: 100,000). Arrow marks the point at which animal was transferred from sea water to the experimental solution. Although the abvisceral and advisceral phases in heart activity typically alternate, owing to rhythmical reversal in the direction of the beat, they have been grouped in this manner for clarity.

animal; and a third period in which the counts were again nearly normal. The advisceral counts were reduced to 1–3 for most of the depressed period but toward the end of the observation time returned to a level higher than that for the fresh animal. In Case B

(1:4,000) the heart stopped after 5 minutes' exposure. About 190 minutes later it was again beating, though irregularly with counts of 3–122 for the abvisceral phase and 3–92 for the advisceral. In a summary of 19 additional cases it was found that the number of

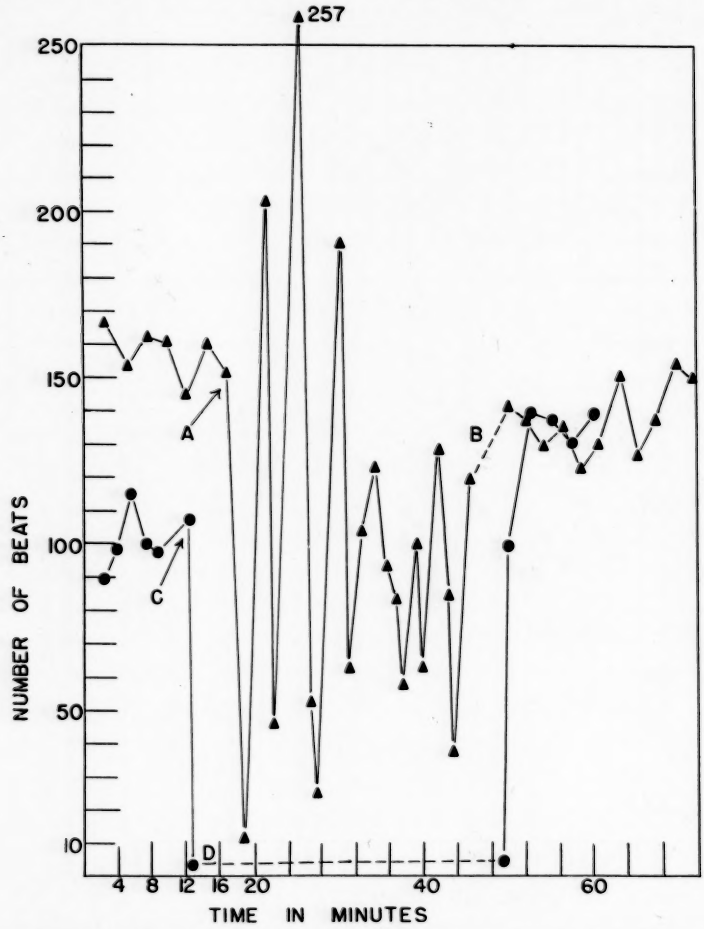

FIG. 2.—Effect of acetylcholine 1: 25,000. *A* and *C* indicate the point where the animal was transferred to acetylcholine; *B*, an interval of 1 hour when the animal was not under observation; *D*, the period when the number of beats in the advisceral phases varied around 1–3 only in consecutive counts. For further explanation see Figure 1.

beats in the abvisceral phase increased to 190–565 during the first 15 minutes' exposure (normal around 145), while the advisceral was reduced to 1–26.

These results suggest that the marked initial increase in the counts of the abvisceral

phase possibly represents a stimulation of the dominancy of one heart center over the other. The frequent temporary hesitations during a long abvisceral dominancy furnish additional support to this suggestion. But at the same time the initial stimulatory period is accompanied by a conspicuous exaggeration of the normal irregularity in the number of beats (Fig. 2)—a state which does not long persist. In strong concentrations acetylcholine has a reversible depressive (paralyzing) effect on both heart centers, while very weak concentrations may increase slightly the length of both phases (Table 1).

If these reactions can be looked upon as a definite stimulatory effect, the action of acetylcholine appears to resemble more closely that on the arthropod heart, where it is an accelerator (Welsh, 1939*a*, *b;* Hamilton, 1939), than on the molluscan heart (Prosser, 1940) or vertebrate heart, where it is always inhibitory. Transfer of an animal from one dish of sea water to another may inhibit beating for a few seconds or may cause a slight depression in both phases with no increase like that described above. Except in cases of marked inhibition of heart action, the animals remained expanded, and circulation continued in body and stolon. By means of temperature elevation Hecht (1918*c*) caused the heart of *Ascidia atra* to start beating from the center, but no instance of this was seen with any of the drugs employed in the present study.

Mecholyl.—This drug is said to have the same properties as acetylcholine and to be more stable. Strong concentrations (1:15,000 to 1:50,000) likewise caused immediate exaggeration of variability and an increase in length of abvisceral phases during the first 10–20 minutes after exposure (Fig. 3). Counts as high as 229–814 (4–13 minutes) and as low as 3 were recorded for different animals (Table 2). Depending to some extent upon concentration, this preliminary stimulatory period was followed by a long period of depressed irregularity in the abvisceral phases. It is evident from this brief summary that the heart reactions to mecholyl resemble those of acetylcholine, i.e., compare Figure 3 with Figure 2.

In Figure 3 (Case D, Table 2) these two periods may be distinguished. In Case C (Table 2) the advisceral counts became consistently higher than the abvisceral, but this was an exception. After 5–20 minutes' exposure, 1:10,000 concentration temporarily stopped the heart, but 10–30 minutes later it resumed activity in the alternating phase. At first reversals were frequent, rate slow and somewhat irregular, and the number of beats small and nearly equal in both phases. In some instances this condition lasted several hours, with counts ranging from 1 to 60, after which the beats approached the normal number. The animals showed no prolonged contraction in nontoxic concentrations of mecholyl, nor was circulation in body and stolon long interrupted.

In the case of the advisceral phases the number of beats immediately became depressed to 1–13, and this condition persisted during the period of increased activity of the abvisceral (Fig. 3, Case D, Table 2). With continued exposure, recovery occurred at approximately the same time as it did in the abvisceral; the counts often slightly exceeded for a time those of the fresh animal (Fig. 2). In Case L (Table 2) during the first 14 minutes' exposure to mecholyl the abvisceral counts were 229, 192, 175, 216, and 166, while the advisceral varied from 2 to 29 with most of the counts fluctuating around the former number until recovery, which came about 135 minutes later. No concentrations of mecholyl, or in fact any of the drugs tested, had other than a depressive action on the advisceral phase. These results suggest (as in the case of acetylcholine) a differential action either direct or indirect upon the heart centers.

Weak concentrations caused no increase in number of beats but only greater irregu-

larity and often slight depression as in the advisceral phase, where counts went as low as 1–6 beats before reversal occurred. In Case B (Table 2) the counts were almost back to normal after 2 hours' exposure, while after $5\frac{1}{2}$ hours the number in the advisceral

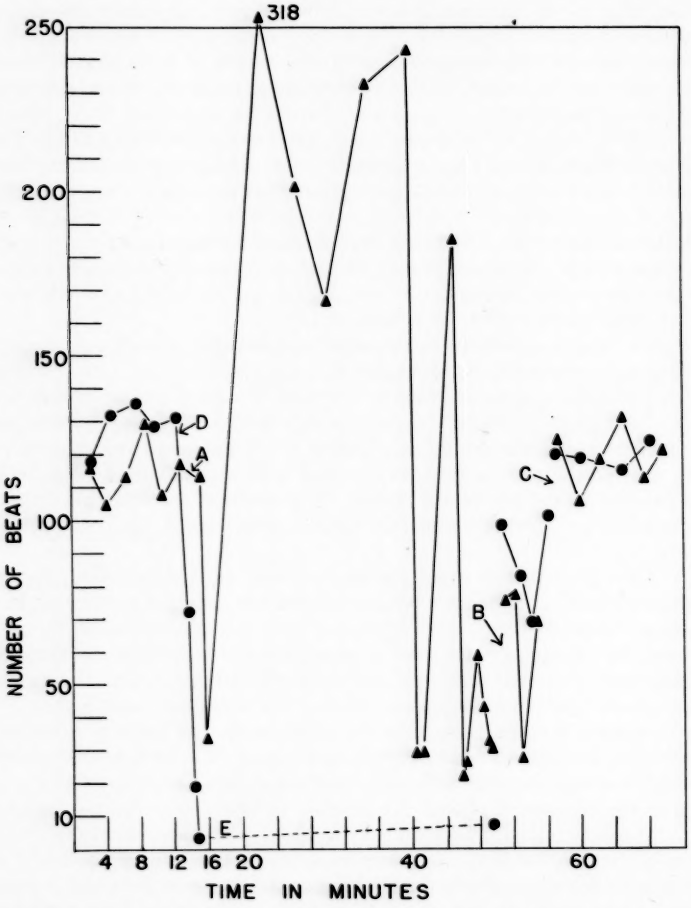

FIG. 3.—Effect of mecholyl 1:50,000. *A* and *D* indicate the point of transference to mecholyl; *E*, a period when the consecutive counts in the advisceral phases varied between 1–8; *B* and *C*, intervals of $2\frac{1}{4}$ and 1 hours, respectively, when the animal was not under observation. For further explanation see Figure 1.

phase often slightly exceeded those in the abvisceral. This latter result has been observed with stronger concentrations, indicating that the discrepancy between the two phases may be reduced or even reversed temporarily. In Case A (Table 2) there occurred progressive depression in both phases accompanied by great irregularity. No

abvisceral count exceeded the normal. After continuous exposure of 3½ hours the abvisceral counts varied around 15–90 and the advisceral 17–38. In another instance the animals were exposed to weak concentrations for 17 hours and then examined. In the 1:300,000 solution the counts in both phases were within the normal range, while in the 1:100,000 solution they were approximately equal, and only the advisceral was within the limits of the normal count. In weak concentrations mecholyl, as well as acetylcholine,

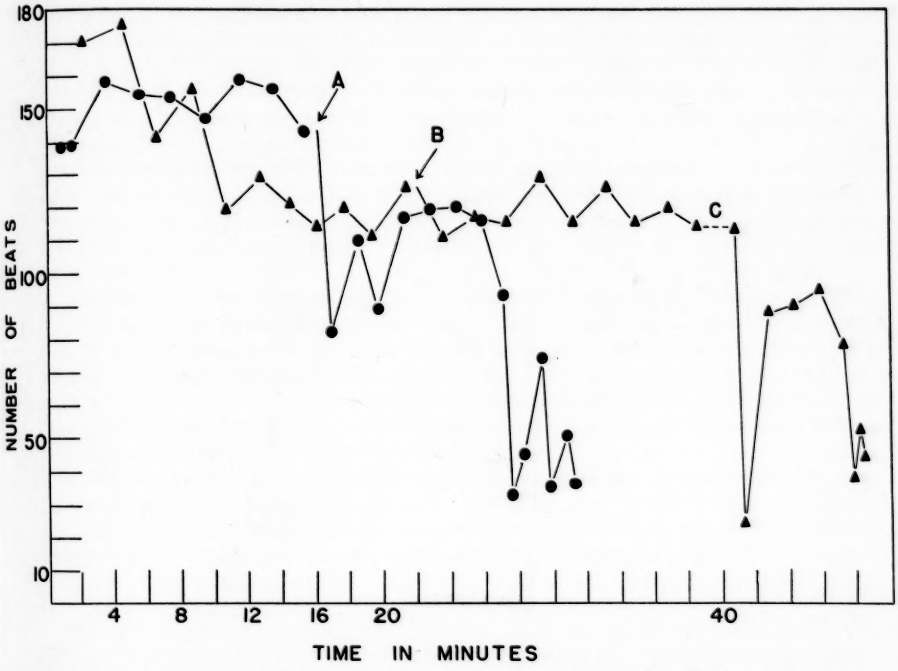

FIG. 4.—Effect of atropine sulphate 1:10,000 (circles) and 1:15,000 (triangles) on variation in the number of heart beats of two animals in consecutive series of abvisceral phases only. At *A* and *B* the animals were transferred to atropine. *C* represents an interval of ½ hour when one animal was not observed.

stimulates the blocked heart to renewed activity and will quicken the activity of a strongly depressed heart.

According to Cohn and Macleod (1939) mecholyl shortens the duration of electrical systole and the refractory period in the frog's heart. It may induce aberrant rhythms, but not heart block.

Adrenalin.—During the effective period of this drug, i.e., about 1½ hours, concentrations of 1:20,000 and 1:25,000 provoked a continuous abvisceral beating of the heart in about 65 per cent of the animals tested, and no reversals to the advisceral phase took place. During this period there occurred at times either a slowing of activity with pulsations originating from the opposite end of the heart without carrying through, or the

heart stopped entirely for 1 or more seconds only to resume beating again in the same direction. By changing the animal to fresh solution hourly the continuity of the abvisceral phase was maintained for at least 5 hours. Reversals often took place at once when the animals were transferred from sea water to the experimental solution during the abvisceral phase, while if the shift was made during the advisceral phase a variable number of reversals over a period of about 10 minutes ensued before the abvisceral phase became continuous. On return to sea water the advisceral phase was initiated at once, or within a few seconds, without regard to the length of time the animal had been exposed

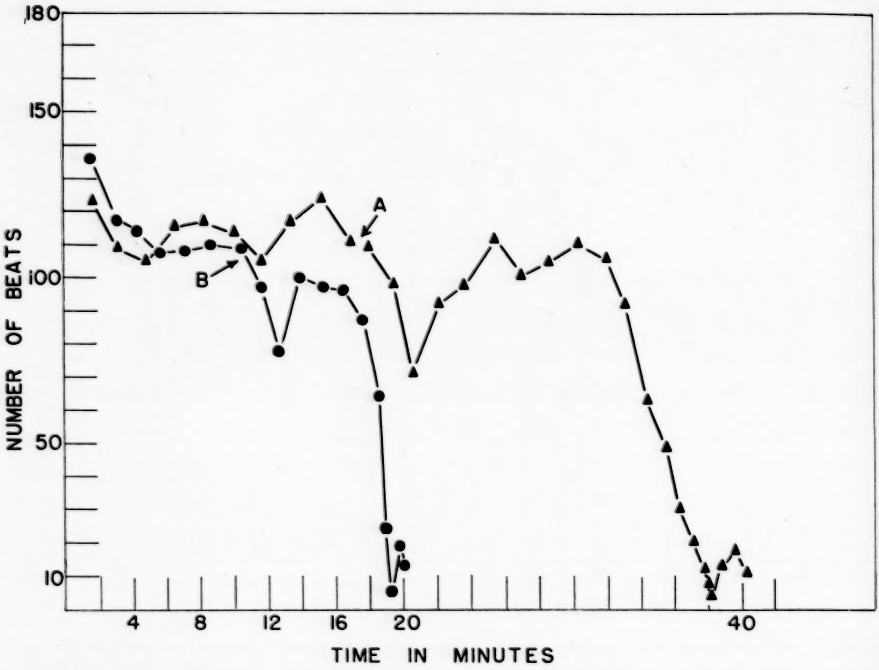

FIG. 5.—Variation in the number of heart beats, in the advisceral phases only, of the same animals as in Figure 4. For further explanation see Figure 4.

to adrenalin. Heart activity was practically normal after several hours in sea water. The action of adrenalin is thus an intensification of that of both acetylcholine and mecholyl in that the length of the abvisceral phase was greatly increased. Size of the animal did not appear to influence the results.

Solutions of 1:50,000 to 1:100,000 did not prevent reversal but increased the irregularity in the two phases as well as the length of some abvisceral phases (2–7 minutes). A concentration of 1:15,000 inhibited dominancy of the abvisceral phase, and the two ends of the heart pulsated without co-ordinated rhythm, and the pulsations failed to carry through. Circulation ceased in body and stolon. These animals recovered when transferred to sea water. If adrenalized animals were transferred to sea water for 3 hours and

then returned to an adrenalin solution of 1:20,000 or 1:25,000 the dominancy of the abvisceral phase was again increased for a short time only (5–10 minutes), while the advisceral phase was merely depressed.

Atropine.—The effect of this drug as shown by the activity of the intact heart was depressive (Welsh, 1939*b*) in both phases. Figures 4 and 5 illustrate the reaction to concentrations of 1:10,000 and 1:15,000. The advisceral phase was depressed more quickly than the abvisceral in each concentration employed. Animals differed slightly in their reactions to atropine. Concentrations of 1:2,500 and 1:5,000 produced some irregularity at once and stopped all heart action within 20 minutes. The general behavior of the heart during this period resembled very closely that caused by lethal doses of acetylcholine, mecholyl, and adrenalin. When transferred to sea water the heart of an atropine-poisoned animal slowly recovered or resumed some slight activity such as twitching at the ends of the tube or an occasional weak pulsation followed by death. Weaker concentrations were progressively less toxic; 1:20,000 and 1:35,000 generally caused considerable irregularity in which a few counts of the abvisceral phase slightly exceeded the normal average, the advisceral counts were reduced to one or several beats after about 25 minutes' exposure, but the heart did not stop, while 1:50,000 was slightly depressive in both phases.

Strychnine.—Weak concentrations (1:100,000 to 1:140,000) were depressive but usually increased the irregularity of heart action, and at times the advisceral outnumbered the abvisceral counts. As stated previously, the latter effect was seldom seen with any of the drugs and then usually during recovery. Concentrations of 1:1,000 to 1:50,000 stopped all heart action within 30 minutes. If the animals were transferred to sea water immediately, they usually recovered. Strychnine did not prevent reversal, nor was there any differential effect on the two heart centers.

Colchicine.—This alkaloid likewise appeared to have merely a depressing action. A concentration of 1:20,000 had no appreciable effect over a short period of time, while 1:5,000 to 1:15,000 progressively decreased the number of beats in both directions. It was less toxic than strychnine but otherwise gave a comparable effect.

DISCUSSION

There is at present no evidence of chemical mediation within the ascidian heart, and, in fact, some investigators cast doubt on the presence of a local nervous system in this organ (Bacq, 1935*b*, *c*, for *Ciona*). According to Hecht (1916, 1918*a*, *b*, *c*) there are no well-defined nerve trunks to the heart of *A. atra*, nor is there even a type of loose nerve net. He concludes that the heart beat originates within the muscle cells and the conduction of the pulsation wave is accomplished by the passage of the impulse across a continuous muscular pathway. Several lines of evidence suggest that the contraction of the ascidian heart may be myogenic—i.e., continued beating of isolated pieces, the effect of temperature (Hecht, 1918*c*), beating of both ends without the transmission of the impulse across the heart (this paper), continued beating after removal or destruction of the intersiphonal ganglion, etc. (cf. Hunter, 1903). According to Prosser (1940, for the clam), this suggests that acetylcholine acts directly on the heart muscle in which there must occur pacemaker, conduction, and contraction processes. Day (1919, 1921) found no evidence of any control exerted by the intersiphonal ganglion in *A. mentula*. The ganglion regulates reflexes in a simple way, and nerves go to siphons, body wall, and viscera (Bacq, 1935; Hilton, 1913). According to Schultze (1901), as reported by Hunter (1903), the heart of *Salpae* is not connected with the central nervous system.

On the other hand, several investigators have described nerve cells and fibers in the ascidian heart. Hunter (1903) found that fibers connect groups of ganglion cells situated on or near the distal ends of the heart tube and the intersiphonal ganglion in *Molgula*. Bipolar nerve cells and fibers run spirally around the heart, a type of loose nerve net. Fedele (1923*a*, *b*; 1927) described a visceral nervous system of tunicates, the role of which is equivalent to that of the vertebrate sympathetic system. Alexandrowicz (1913) states that he saw nerve cells, but he gives no figures.

Concerning the phenomenon of heart reversal—an ascidian characteristic—recent studies seem to agree that it is the result of a difference in activity of a center at each end of the heart tube and not to the "back pressure" assumption of some of the earlier workers. One of these centers may be more powerful than the other in certain ascidians, at least the number of beats in one direction is larger than in the other. Hecht (1918*c*) speaks of the "dominance of pacemakers," while Bacq (1935) refers to "centers of automatic stimulation," which work periodically and rhythmically. The center which is dominant at a given moment is the one possessing the greatest frequency. Beating is reversed when the chronotropism of the resting center becomes superior to that of the functioning one.

In the present study it has been found that acetylcholine, mecholyl, and especially adrenalin cause a lengthening of the dominant period of the abvisceral phase above that characteristic of the quiescent animal and always reduce the length of the advisceral phase; while atropine, strychnine, and colchicine depress both phases in relation to the concentrations employed. If the intersiphonal ganglion does not exert much, if any, controlling action on the heart, the primary effect of the stimulating drugs may be directly upon the heart centers and their pacemaker mechanisms. If so, the drugs must penetrate immediately because their effects are detected at once, followed by quick recovery in sea water. It is probable that the conductile and contractile mechanisms in the heart are relatively unimpaired at weak concentrations. On the other hand, strong concentrations, which eventually reduce heart action to unco-ordinated pulsations at the ends, may affect not only the pacemaker mechanisms so that no general wave of excitation is set up but possibly the conductile and contractile mechanisms as well. This may especially be true of atropine, since in an atropinized heart which has stopped beating the pulsations are renewed only at each end after transfer to sea water, and this partial revival is followed by eventual death.

Among other effects observed in this study may be mentioned the following: (1) the period of increased abvisceral dominance is often accompanied by an intensification of the typical irregularity in the number of beats in consecutive abvisceral counts; (2) this period is generally followed by one of marked decrease in the number of beats for the abvisceral phase; (3) transfer of the normal animal from one container of fresh sea water to another may result in a slight and temporary depression in number of beats, but it never causes any increase in abvisceral number; (4) the increase is not caused merely by the reduction in the advisceral number, because this depression takes place independently while the abvisceral phases are passing through both stimulated and depressed periods; (5) strong concentrations cause only depression; (6) the only effect upon the advisceral phase is one of depression; (7) during one of the extra-long abvisceral phases the heart may almost cease beating for a moment with simultaneous contractions starting from the opposite ends, but in every instance the contractions fail to be carried through and the abvisceral beat is then continued; (8) these effects are usually quickly reversible,

providing the exposure has not been too long or the heart stopped. Consideration of these various points has led to the conclusion that the provoked increase in the abvisceral counts represents a case of stimulation of the impulses liberated by the pacemaker mechanism rather than a weakening or depression of the center at the opposite end of the heart.

The experimental studies of Hunter (1903) on *Molgula* cast doubt on the absence of cardiac innervation in ascidians. He observed effects quite similar to those described in this paper, i.e., irregularity of rhythm, lack of co-ordination between the ends, and long pulsation periods in only one direction in the isolated heart, after cauterization of the ganglionic region. Furthermore, after cauterization of the posterior end of the ganglion and the anterior region of the dorsal nerve cord there was provoked a series of interfering double beats. In the absence of the ganglion, strychnine had no effect. Hunter obtained no effect by electrical stimulation. Schultze (1901, reported by Hunter) considered that the abnormally long periods of rhythmical pulsations in one direction only are a sign of approaching death.

The presence or absence of acetylcholine in ascidian tissues remains to be determined, as well as a possible liberation of this substance during nerve stimulation. Bacq (1935*c*) has found choline esterase, a suggestive discovery. Since the amount of nerve tissue in *Perophora*, or any ascidian, is not large, the amount of acetylcholine produced may be so minute as to be quickly destroyed and hence fail to appear during analysis. The stimulating action of acetylcholine is possibly due to its muscarine-like effect, since preliminary results, to be reported later, show (1) an antagonism between atropine and the action of acetylcholine and (2) that stimulation does not follow treatment with weak concentrations of atropine. Comparison of the effects on the *Perophora* heart with those caused by similar drugs on the hearts of animals said to have a local nervous structure which is cholinergic suggests furthermore that (*a*) the heart of this ascidian may have a nervous structure of its own and (*b*), if so, a cholinergic type of chemical mediation occurs (cf. Cannon, 1939). This suggestion is supported by certain other preliminary studies which indicate that eserine also increases the number of abvisceral beats, lowers the threshold of sensitivity to acetylcholine, and increases the length of the response. The fact that the heart reacts to weak concentrations of acetylcholine and especially adrenalin leads to the inference that it is innervated (cf. Marrazzi, 1939). According to Brown (1937), the potentiating effect of eserine is strong evidence of cholinergic transmission.

SUMMARY

1. The question of chemical mediation in the function of the heart of ascidians has been attacked by exposing the compound ascidian, *P. viridis*, to acetylcholine, mecholyl, adrenalin, atropine, strychnine, and colchicine. Indicators of the effects produced upon heart activity consisted in the effect on heart reversal, changes in the number of beats in the two different phases, length of these phases, length of the rest periods between reversals, and frequency of the beat.

2. Dominance of the abvisceral phase is prolonged by acetylcholine, mecholyl, and especially adrenalin, while in all instances the advisceral phase is strongly depressed. Strong concentrations inhibit the dominance of both centers and eventually stop the heart. With frequent changes of the experimental solution adrenalin causes the heart to beat in one direction for hours. Atropine, strychnine, and colchicine depress both phases.

3. Consideration of the effects produced by these various drugs has led to the conclusion that the provoked increase in the length and variability of the abvisceral phases represents a stimulation of the effective heart center and its pacemaker mechanism. Only stronger or depressive concentrations may effect the conductile and contractile mechanisms of the heart.

4. Although there is some question in the literature as to whether the ascidian heart is innervated or even possesses a local nervous system of its own, comparison of these results with those involving the use of similar drugs on the hearts of other animals suggests that a nervous system may be present and, if so, that it may possess a cholinergic type of chemical mediation. Furthermore, the results appear to resemble more closely those secured with certain invertebrate hearts (especially arthropod) than with either molluscan or vertebrate hearts.

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STUDIES ON THE METABOLISM OF MARINE INVERTEBRATE TISSUE
I. RESPIRATION OF THE MIDGUT GLAND OF THE KELP CRAB (PUGETTIA PRODUCTA)

(Four figures)

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IN CONTRAST to the abundance of data in the literature on the respiration of excised vertebrate (particularly mammalian) tissue stands the scarcity of similar data on other groups. The work of Hopkins (1930), Chapheau (1932), and Fox and Wingfield (1937) may be cited as contributions in this latter field. The present study was designed to provide additional data on the respiration of marine invertebrate tissue *in vitro*. It is, however, part of a larger series of studies in progress at Hopkins Marine Station which is intended to provide as much information as possible concerning the physiology of the tissues, organs, and organ systems, together with relevant features of the natural history, of a number of the marine forms occurring in Monterey Bay, California. The kelp crab (*Pugettia producta*) was chosen as the first animal to be studied in this program because considerable biometric data relating to this form have been collected by one of us (Weymouth). The midgut gland was selected as the first organ for investigation because of its functional importance and because it is relatively large among the organs of the kelp crab and constitutes a relatively constant fraction of total body weight through the various stages of the molt cycle (Drach, 1939).

The midgut gland is an organ unique to and characteristic of the Crustacea. Since it is without homology in the mammal, the term "liver" and "hepatopancreas" are unjustified. Because it develops from the midgut, the term "midgut gland" identifies it without chance of error and with no misleading implications as to function. An excellent account of the history and present knowledge of the structure and functions of the midgut gland is given by Drach (1939).

MATERIAL AND METHODS

The work reported was done at Hopkins Marine Station during the summers of 1938 and 1939. Crabs were kept in well-aerated, running water provided with kelp and used within 4 days of capture in the near-by kelp beds. Sixty-one animals were used (28 males and 33 females), selected to give a graded series in respect to carapace length (measured from the rostral notch to the posterior margin of the carapace) and live weight. The range of these measurements was 1.30–9.68 cm. and 0.50–275 gm., respectively.

LENGTH-WEIGHT RELATIONS

These specimens, added to others studied in respect to life-history, made available a series of lengths and weights of 742 animals (366 males and 376 females). Plotted on a

double logarithmic scale, these data showed a linear relation between length and weight except for individuals (of both sexes) below 2.25 cm. and males over 7.25 cm. in length. Excluding these and combining data for both sexes (which showed no significant differences within this range), we found that the data for 209 males and 228 females were fitted by the conventional formula $W = aL^b$. Values for the constants a and b are given in equation (1):

$$\log W = \log 0.6597 + 3.0097 \log L . \qquad (1)$$

This close approximation to the cubic relation indicates that within this size range there is little change of form. The large males are heavier because of the great size of the chelae, which show rapid relative growth after sexual maturity.

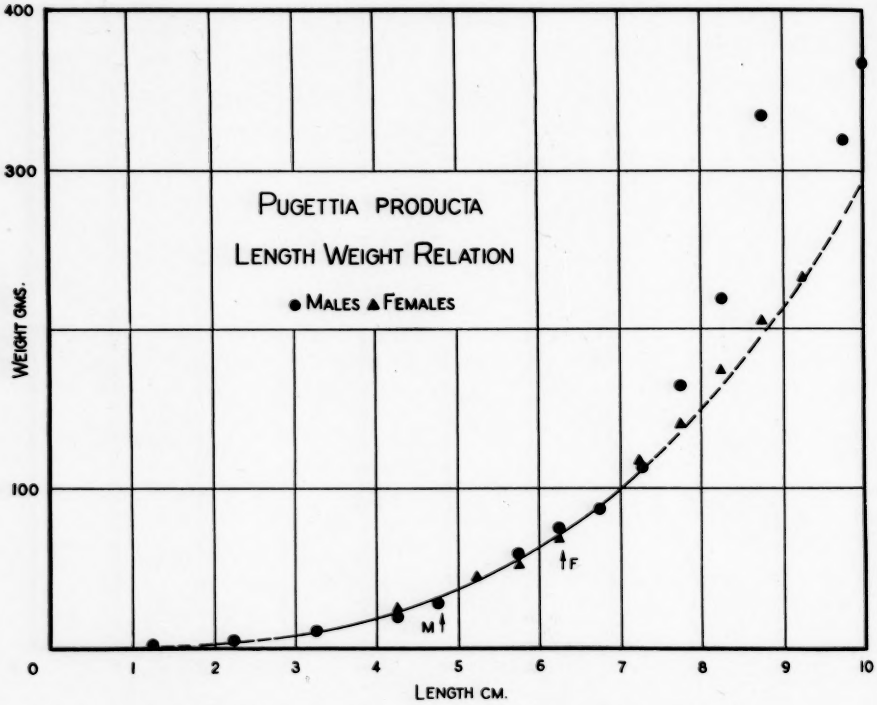

FIG. 1.—Length-weight relations of 366 male and 376 female kelp crabs

The data, together with the fitted curve relating weight to length, are given in Figure 1 and Table 1. The columns of the table give, in order, the midpoints of the length classes in centimeters, the number and mean weights of the males and females in these classes, and, for the range of body size in which equation (1) is applicable, the weights calculated from this equation.

SIZE AT SEXUAL MATURITY

Length and weight data at sexual maturity are available for 723 crabs. The criteria for maturity in the females were the presence of eggs and the shape of the abdomen, which at the imaginal molt undergoes a marked widening and increase in convexity. The criteria for males are rather less certain. Those used were the size of the gonads (relatively large during the summer, the breeding season for this form) and the presence of large white vasa efferentia. Of 432 females, 50 per cent were mature at a length of 6.30 cm. (25 per cent at 5.00 and 75 per cent at 7.15 cm.). Of 291 males, 50 per cent were mature at a length of 4.80 cm. (25 per cent at 4.10 and 75 per cent at 5.65 cm.).

TABLE 1

LENGTH-WEIGHT DATA FOR *Pugettia producta*

Both Sexes	Males		Females		Both Sexes
Length (cm.)	Number	Weight (gm.)	Number	Weight (gm.)	Calculated Weight (gm.)
1.25	25	1.06	21	0.79	.....
1.75	63	1.88	70	1.81	.....
2.25	61	3.96	57	3.75	.....
2.75	28	6.42	24	6.38	6.01
3.25	30	10.17	30	10.42	9.94
3.75	26	15.14	28	15.09	15.29
4.25	21	21.37	24	22.60	22.28
4.75	28	29.91	25	30.55	31.15
5.25	24	42.08	17	42.50	42.10
5.75	16	57.81	21	52.76	55.36
6.25	10	74.10	12	69.58	71.14
6.75	14	87.86	9	89.27	89.66
7.25	12	114.79	9	116.39	111.15
7.75	2	165.00	10	139.75	.....
8.25	1	220.00	7	174.64	.....
8.75	3	335.00	9	205.00	.....
9.25	0	.....	3	231.67	.....
9.75	1	320.00	.....	.....	.....
10.00	1	369.00	.....	.....	.....
Total number of animals.....	366	.....	376	.....	.....

Of the 61 crabs used in studies on tissue respiration, all but 4 were hard shelled (probably stage *C* of Drach, 1939). Of the hard-shelled group, only 4 were about to molt, as indicated by the presence of an inner soft shell (probably stage *D* of Drach). Since the respiration data on these 8 animals did not appear out of line with the rest, they were included in the analysis of results.

PREPARATION OF TISSUE SLICES

In the earlier experiments, pieces of midgut gland were excised and placed in filtered, aerated sea water. Paired samples of suitable size for the respirometer vessels (50–100 mgm. wet weight) were dried rapidly by blotting with hardened filter paper and quickly

weighed on a microtorsion balance. One of each pair was then placed in a respirometer, the other in a crucible, which was then dried to constant weight in an electric oven at 105° C.

However, it was found that midgut gland in sea water rapidly takes up water to the extent of about 15 per cent of its initial wet weight. Thus wet weights after immersion were not the same as those obtaining *in vivo*. Nevertheless, the procedure described above made possible the calculation of dry weights of the samples in the respirometer vessels because these were removed from sea water at the same time as the samples for drying. In order to determine wet weights more accurately in the later experiments, the excised tissue was quickly blotted dry and placed in a Petri dish which was humidified by sticking a piece of filter paper saturated with sea water to the under surface of the cover. Then paired samples were taken as before.

The problem of tissue-slice thickness (cf. Dixon, 1934) did not arise, because after a few minutes of shaking (during the period of thermal equilibration) the pieces of midgut gland were thoroughly dispersed in the medium which took on the appearance of a "soup." The tubules which did not break up were very slender—certainly thinner than requisite for adequate diffusion of oxygen. Thirty to 40 minutes usually elapsed between excision of tissue and the beginning of measurement of respiration (end of the thermoequilibration period).

MEASUREMENT OF RESPIRATION

Oxygen consumption was measured in conventional Warburg vessels, which were shaken in a constant-temperature bath at 15° ± 0°.01 C. (selected because the temperature of sea water in the animal's natural habitat was about 15°). The rate of shaking was 110 complete oscillations per minute, the amplitude 8 cm. The suspension medium was filtered sea water. The gas phase was oxygen. The central wells of the respirometer vessels contained 5 per cent NaOH (and wicks of Whatman No. 40 filter paper) for absorption of CO_2. Twenty minutes of thermoequilibration preceded each run. Oxygen uptake was followed at 20-minute intervals for at least 2 hours.

The symbol Q_{O_2} is used to denote the rate of oxygen uptake in cubic millimeters at normal pressure and temperature, per milligram dry weight per hour.

EXPERIMENTAL

Suitable conditions for the measurement of the Q_{O_2} of midgut gland must be established before the relationship of such respiration to other factors can be investigated. As far as we know, this has not been done for crustacean tissue. Accordingly, this section consists of two parts, the first concerned with method, the second with applications.

PART I

1. *Studies on the variability of manometer readings.*—To determine the lowest limit of variability possible under the conditions of our experiments, two sets of manometers (fourteen instruments) were run as thermobarometers (cf. Dixon, 1934, p. 15). This was done on a day when considerable change in barometric pressure was expected and, in fact, occurred. Temperature was constant at 15° ± 0°.01 C. The results are given in Table 2.

The variability reported in Table 2 is attributable to factors that are always operative, such as irregularities in movement of manometer fluid, errors in reading, and the like.

When tissue fragments are placed in the respirometer vessels, it is clear that there will be additional variability. The view that the data of Table 2 indicate our lowest limit of variability is supported by the uniformity of the variability in the readings at different times. The greatest difference—that between the standard deviation at 2 hours and the standard deviation at 3—might be duplicated or exceeded once in four times by chance in samples of this size drawn from a series in which no real difference in variability existed. This indicates that the absolute variability is constant. Since this is so, it follows that the percentage variability decreases with increase in the manometric excursion. This is shown by the decrease in coefficient of variability of the 2- and 3-hour readings as compared with that of the 1-hour readings.

TABLE 2

STATISTICAL ANALYSIS OF DATA OBTAINED WHEN FOURTEEN WARBURG MANOMETERS WERE RUN AS THERMOBAROMETERS

Temperature, 15° ±0°.01 C. Rate of shaking, 110 complete oscillations per minute. Amplitude, 8 cm.

Time of Reading (Minutes)	Manometric Range (Cm.)	Excursion Mean (Cm.)	Standard Error of Mean (Cm.)	Standard Deviation (Cm.)	Standard Error of Standard Deviation	Coefficient of Variation
60.....	1.17–1.40	1.31	0.020	0.075	0.01369	5.73
120.....	1.85–2.12	2.00	0.017	0.062	0.01132	3.11
180.....	2.16–2.43	2.29	0.022	0.083	0.01515	3.62

a) Goodness of check.—An example of the closeness of check obtained between values of total oxygen consumed, N.P.T., per milligram dry weight when fragments of midgut gland from one animal are placed in each of two respirometer vessels is given in Table 3.

To facilitate comparison with the data of Table 2, the data listed in Table 3 are calculated in terms of centimeters of manometer excursions as well as in terms of percentage differences. It is clear that the "lower-limit" variability shown in Table 2 is a component of the variability shown in Table 3, which may be called "total variability," since it includes variability attributable to tissues and filling operations as well as the variability of readings (given in Table 2). If the variabilities are expressed in terms of the standard deviations as variances, the following equation relates the total variability to the several variabilities of which it is composed:

$$S_T^2 = S_I^2 + S_{II}^2 ,$$

where

S_T = "Total" standard deviation from Table 3 = 0.322 ,

S_I = Standard deviation from Table 2 = 0.07216 (average) ,

S_{II} = Standard deviation attributable to tissues, filling, etc.

From this equation it can be calculated that $S_{II} = 0.314$ and that the "lowest-limit" variance, (S_I^2), is about 5 per cent of the total variance, (S_T^2), when manometer excur-

sions are of the order of 5 or 6 cm. Thus it is evident that under these conditions the variability of reading, etc., is rather small compared with that of the tissue samples and filling operations.

The goodness of check may be described as follows: Expressed on a relative basis, the average pair of check readings in Table 3 differ by 5.97 per cent of the higher value. The standard deviation of such percentage differences is 3.80. Hence in about 68 per cent of the cases the lower reading of a pair will differ from the higher one by not more than 9.77 per cent and not less than 2.17 per cent of the higher value.

TABLE 3

VARIABILITY OF DUPLICATE MEASUREMENTS OF RESPIRATION

Animal No.	O_2 Consumed, in Cubic Millimeters, N.P.T. per Milligram dry weight during First Hour		Percentage Difference between I and II (Higher Value = 100)	Difference between I and II in Centimeters on Manometer Scale*
	I	II		
25	1.342	1.538	12.7	0.61
27	1.468	1.621	9.4	0.48
29	1.508	1.435	4.8	0.23
30	1.360	1.259	7.4	0.32
31	1.880	1.940	3.1	0.19
42	4.000	4.240	5.7	0.75
43	2.880	2.575	10.6	0.95
44	2.415	2.515	4.0	0.16
45	2.045	2.000	2.2	0.07
46	3.175	2.885	9.1	0.90
48	2.900	2.650	8.6	0.78
53	2.555	2.500	2.2	0.17
54	2.360	2.580	8.5	0.34
55	2.870	2.890	0.7	0.03
56	2.295	2.310	0.6	0.02

Standard deviation of percentage differences = 3.80,
Standard error of mean of percentage differences = 0.981,
Standard deviation of differences in centimeters = 0.322,
Standard error of mean of differences in centimeters = 0.0831.

* These values vary with the manometer used.

2. *Determination of suitable quantitative relations between the amounts of tissue and of suspension medium.*—It is well established that the ratio

$$\frac{\text{Parts tissue (by weight)}}{\text{Parts suspension medium (by weight)}},$$

hereinafter called the "tissue/medium ratio," may modify the Q_{O_2} of tissue. High values tend to lower Q_{O_2} through interference with the diffusion of oxygen (Dixon, 1934), while low values may involve decrease in Q_{O_2} due to loss of soluble respiratory catalysts and substrate (Krebs and Eggleston, 1938). Thus a suitable tissue/medium ratio must be found if values of Q_{O_2} representative of the tissue are to be determined. The data given in Table 4 are the results of experiments designed for this purpose.

It is shown in Table 4 that Qo_2 remains practically constant as the tissue/medium ratio increases from $\frac{1}{20}$ to $\frac{1}{10}$ and then declines with further increase in this ratio. In all later experiments the tissue/medium ratio lay in the range of constant Qo_2.

3. *Effect on Qo_2 of variation in the time between excision and measurement.*—It has been shown by Knowlton and Hines (1934) and by Field, Belding, and Martin (1939) that Qo_2 is a function of the time between death of the animal and experimental observations for many mammalian tissues under the usual conditions of experimentation *in vitro*. The experiments described below were designed to assess the importance of this relationship under our working conditions.

Three large animals were used, so that enough tissue would be available. The midgut gland was excised as rapidly as possible and placed at once in a Petri dish containing a small amount of filtered sea water. To minimize loss of diffusible respiratory catalysts

TABLE 4

EFFECT OF VARIATION IN TISSUE/MEDIUM RATIO ON Qo_2 OF MIDGUT GLAND

(Animal No. 10, male, weight 92 gm., length, 6.80 cm.)

Number of Vessels	Tissue Wet Weight in Milligrams	Tissue/Medium Ratio	Qo_2* as Percentage of Value when Tissue/Medium Ratio = 100
2	100	1:20	100
1	100	1:10	96
1	200	2:10	85
1	299	3:10	63

* Sixty-minute Qo_2.

and substrate, the tissue/medium ratio in the Petri dishes was made very large. Respirometer vessels were filled at 30-minute intervals, so that measurement of oxygen consumption began 30, 60, 90, and 120 minutes after excision. From these measurements the data of curves *A*, *B*, *C*, and *D*, respectively, in Figure 2 were calculated. Time values on the *X*-axis in Figure 2 represent reading times and not times after excision. Each run was made in triplicate.

A value of 100 was assigned to the mean Qo_2 of the earliest run at the 15-minute interval. Subsequent values of Qo_2 in the other three runs on the same animal were expressed in terms of their relative magnitude on this basis. In each case Qo_2 decreased with time. The next step was to average these relative Qo_2 values at corresponding reading times for the 3 animals. The resulting means constitute the values plotted in Figure 2. Thus each point on each curve of Figure 2 is based on nine measurements of respiration (three per animal).

It should be noted that the first point on curve *B* corresponds to the second point on curve *A* in time measured from excision (both are 45 minutes after excision); similarly, the first point on *C* corresponds to the second on *B*, and so on.

It is shown in Figure 2 that up to 60 minutes after excision the capacity for respiration is maintained better on standing in the Petri dish than in the respirometer vessels. Thus

the 45-minute Qo_2 on curve *A* is 83.5, and the 15-minute Qo_2 on *B* is 92.4, although both correspond to a time of 75 minutes after excision. This effect disappears on longer standing, because the 45-minute Qo_2's on *B* and *C* are about the same as the 15-minute Qo_2's on *C* and *D*, respectively.

The portions of curves *A*, *B*, *C*, and *D* which appear in Figure 2 are hyperbolas and were fitted by the following equations (where t denotes time):

$$A.\ \log Qo_2 = \log 100.1 - 0.266 \log t,$$

$$B.\ \log Qo_2 = \log 93.1 - 0.269 \log t,$$

$$C.\ \log Qo_2 = \log 81.4 - 0.257 \log t,$$

$$D.\ \log Qo_2 = \log 69.9 - 0.228 \log t.$$

While further work is necessary to determine the factors responsible for these relations, a few comments can be made on the basis of evidence now available. The better

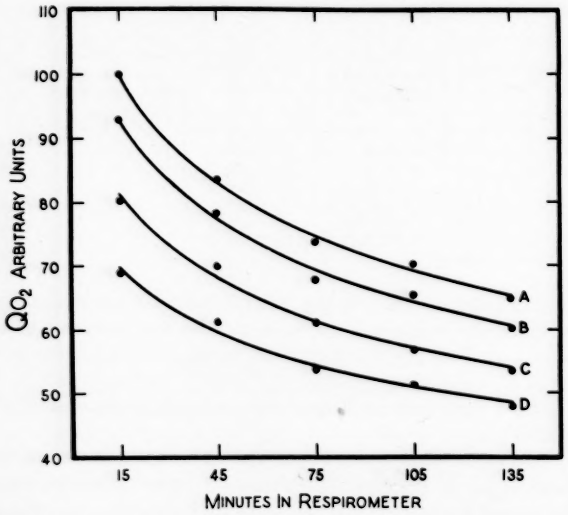

FIG. 2.—Qo_2 of midgut gland as a function of time after excision (see text for further description)

maintenance of the capacity for respiration in tissue standing 60 minutes as compared with that standing 30 minutes and in the vessels 30 minutes (represented by curves *A* and *B*, respectively) is probably not attributable to a better supply of oxygen, since the conditions of oxygen supply were more favorable in the vessels. If it is an "oxygen-debt" effect (cf. Hill, 1926), the importance of accumulation of oxidizable substrate becomes less than that of some other factor after 60 minutes. It seems probable that an important factor was the more limited loss of diffusible respiratory catalysts and substrate in the

Petri dish, where the tissue/medium ratio was high. The subsequent disappearance of this effect (*C* and *D*) may be attributable to damage to tissue cells consequent upon prolongation of the period of relative anoxia.

However, no matter what factors may have determined the time course of Qo_2, it is clear that the time interval between excision and the measurement of respiration must be kept constant in any series of experiments designed to show the relation between midgut gland Qo_2 and other factors. In subsequent work this period was maintained at 30–40 minutes.

4. *Possible metabolic gradients in the midgut gland.*—Whether or no metabolic gradients in respect to oxygen consumption obtain in the midgut gland is not known. To answer this question, the Qo_2 of anterior, median, and posterior regions of the gland were determined, with tissue from a single large animal (No. 22, female; length, 9.30 cm.; weight, 268 gm.). These were found to average 1,628, 1,509, and 1,514, respectively (each value is the mean of four determinations). These differences cannot be considered significant, since the largest might be expected once in four times in a series of constant variability. However, it became our practice to use tissue from the anterior and median regions except in the case of small animals.

PART II

1. Qo_2 *of midgut gland as a function of body size (age).*—A negative correlation between age and basal metabolism (DuBois, 1936; Pearce, 1936) and between age and the Qo_2 of various tissues (Pearce, 1936; Field, Tainter, Martin, and Belding, 1937) has been shown for a number of vertebrates. For the invertebrates, Crismon and Hall (unpublished) found a decrease in the rate of oxygen consumption of the intact kelp crab with increase in body size, and Hopkins (1930) and Chapheau (1932) have shown that the respiratory rate of excised tissue from the clam (*Venus mercenaria*) and the oyster, respectively, decreases with increase in body size. In consonance with these observations, it is shown in Table 5 that the Qo_2 of midgut gland from the kelp crab also decreases with increase in body size.

Although the form of the regression of body size on age is not known for the clam, oyster, or kelp crab, it cannot differ radically from the curvilinear regression known for related forms, such as the razor clam (*Siliqua patula;* Weymouth, McMillin, and Rich, 1925) and the Pacific edible crab (*Cancer magister;* MacKay and Weymouth, 1935). Therefore the negative correlations between respiration and body size reported above imply a negative regression of respiration on age, although the exact form of the regression cannot be stated.

The regression of Qo_2 on carapace length and on log body weight proved to be approximately linear, whereas the regression on body weight was curvilinear. Hence, for simplicity, log body weight was chosen as a variate in statistical analysis instead of body weight. It is shown in Table 5 that there is a high negative correlation between Qo_2 and both carapace length and log body weight (which in turn are correlated with age). By the use of Fisher's *Z*-scale (Treloar, 1939, p. 158) it was found that there is less than one chance in one thousand that the true correlation of Qo_2 with carapace length is less than 0.66, while the correlation with log body weight is still more reliable. Similarly, it was found that all the correlations in Table 5 were significant. However, in no instance is the difference between them significant, although the coefficients of correlation involving log body weight are a little higher. Perhaps in a larger series the coefficient of correla-

TABLE 5

DATA BEARING ON THE CORRELATION BETWEEN BODY SIZE AND Q_{O_2} OF THE EXCISED MIDGUT GLAND OF THE KELP CRAB AT 15° C.

(Carapace length in centimeters and log body weight in grams)

Statistical Data	Relation between Carapace Length (x) and Q_{O_2} (y)			Relation between log Body Weight (x) and Q_{O_2} (y)		
	Males	Females	Both Sexes	Males	Females	Both Sexes
Number of animals	28	33	61	28	33	61
Range in carapace length (x)	1.30–9.68	1.80–9.30	1.30–9.68			
Mean of carapace length ($\bar{x}$)	4.91	6.08	5.54			
Range in log body weight (x)				−0.3010 to 2.4393	0.2304 to 2.4281	−0.3010 to 2.4393
Mean log body weight ($\bar{x}$)				1.3438	1.6901	1.5312
Range in Q_{O_2} (y)	0.63–3.80	0.47–3.98	0.47–3.98	0.63–3.80	0.47–3.98	0.47–3.98
Mean Q_{O_2} ($\bar{y}$)	1.92	1.57	1.73	1.92	1.57	1.73
Standard deviation of x	2.265	2.382	2.401	0.759	0.640	0.718
Standard deviation of y	0.804	0.784	0.812	0.804	0.784	0.812
Coefficient of correlation, r_{xy}	−0.863	−0.815	−0.845	−0.863	−0.862	−0.867
Regression coefficient, b_{yx}	−0.306	−0.268	−0.285	−0.914	−1.055	−0.979
Intercept, a	3.420	3.201	3.311	3.144	3.352	3.228
Standard error of r_{xy}	0.0491	0.0594	0.0369	0.0491	0.0454	0.0320
Standard error of estimate, $S_{y \cdot x}$	0.406	0.454	0.434	0.406	0.397	0.405

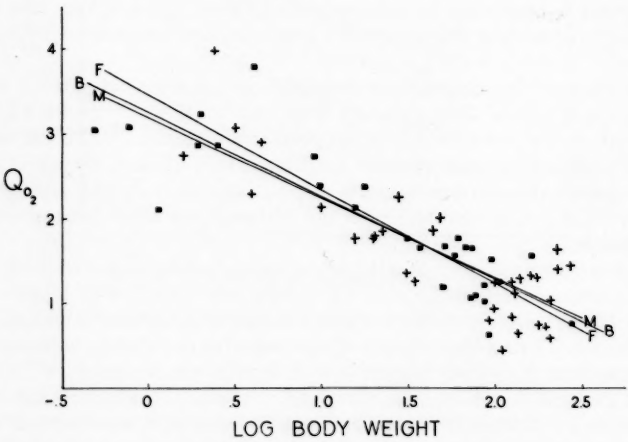

FIG. 3.—Scatter graph, showing Q_{O_2} as a function of log body weight. Black squares denote males; plus signs, females. Lines *MM*, *FF*, and *BB* are regression lines of Q_{O_2} on log body weight for males, females, and both sexes, respectively.

tion between Qo_2 and log body weight would be significantly higher than that between Qo_2 and carapace length, since Qo_2, a rate per unit weight, might be expected to correlate better with relative changes in weight than with a linear dimension like carapace length. There was no significant difference between the regression coefficients for the two sexes in either case, indicating that the Qo_2 of midgut gland does not vary with sex.

A scatter graph illustrating the relation between Qo_2 and log body weight is presented in Figure 3.

The equations for the linear regression lines of Qo_2 on carapace length and on log body weight are as shown in the accompanying table.

Qo_2 ON CARAPACE LENGTH (L)

1. Males	$Qo_2 = 3.420 - 0.306L$
2. Females	$Qo_2 = 3.201 - 0.268L$
3. Both sexes	$Qo_2 = 3.311 - 0.285L$

Qo_2 ON LOG BODY WEIGHT (W)

1. Males	$Qo_2 = 3.144 - 0.914 \log W$
2. Females	$Qo_2 = 3.352 - 1.055 \log W$
3. Both sexes	$Qo_2 = 3.228 - 0.979 \log W$

2. *Qo_2 of midgut gland as a function of temperature.*—Many data are available in the literature on the influence of temperature on various metabolic activities of animals (Bělehrádek, 1935; Burton, 1936; Heilbrunn, 1937). However, the effect of temperature on the Qo_2 of crab tissue does not appear to have been studied. For this purpose 6 animals, 3 males and 3 females of fairly uniform body size, were selected.

Measurements of midgut gland respiration were carried out at graded temperatures, 10°, 15°, 20°, 25°, and 30° C., all constant to $\pm 0.°01$ C. Runs on tissue from a given animal were made concomitantly. Owing to limitation in equipment, simultaneous runs were possible only at two temperature levels for the first 4 animals (10° and 15° and 15° and 20°), but through the kindness of Dr. van Niel it was possible to run the last 2 animals at three levels (15°, 25°, and 30°). In every case three or four measurements were made at the temperature indicated.

Assessment of the effect of temperature on the Qo_2 of midgut gland is complicated by the decrease of Qo_2 with time (cf. Fig. 2), which varies at different temperatures, being least marked at 10° C. and most at 30° C. Accordingly, the data are presented in Figure 4 in such a way as to illustrate the relation of Qo_2 to both these factors.

Since runs were made at 15° C. on all animals, these data were used as a basis of reference. A value of 100 was assigned to the Qo_2 at 15° and 10 minutes, relative values of Qo_2 for other times and temperatures were calculated, and the average values so obtained are plotted in Figure 4.

The curves drawn in Figure 4 were fitted to the data by Fisher's method of orthogonal polynomials (Snedecor, 1940, p. 324). All these can be represented by the empirical equation (parabola)

$$Qo_2 = a + bT + cT^2, \qquad (2)$$

where T denotes temperature and Qo_2 is "relative Qo_2."

Relative Q_{O_2}'s at 25° and 30° are connected by broken lines in Figure 4 because the relations obtaining in this range cannot be determined from our data. That the optimum temperature exceeds 25° is probable, both because of the smooth fitting of the curves between 10° and 25°, which does not suggest discontinuity, and because numerous observations by one of us (Weymouth, unpublished) have shown that the thermal optimum for many processes in the Monterey Bay invertebrates exceeds 25° C. It is suggested that these curves would show rounded peaks with rapid catacrotic decline (cf. Pantin, 1924, Figs. 2 and 3) if sufficient data were available to describe them more fully.

a) Regularity.—It is evident from inspection that the processes represented are orderly with respect to time and temperature, so that, in general, inferences drawn from any one curve are supported by the entire body of data.

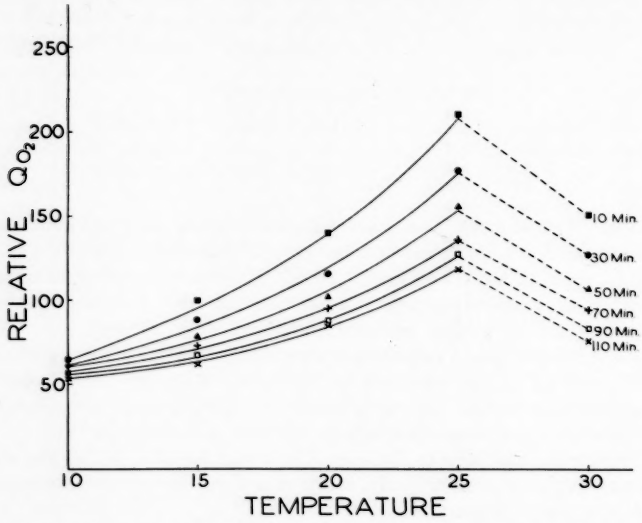

FIG. 4.—Q_{O_2} of midgut gland as a function of environmental temperature and time (see text for further description).

b) Q_{O_2}-temperature relations in the range 10°–25° C.—For any finite increase in T, the corresponding increase in Q_{O_2} is given by equation (3),

$$\overline{\Delta Q}_{O_2} = \overline{\Delta T}(b + 2cT) + c\overline{\Delta T^2}\,. \tag{3}$$

Since the constants a, b, and c are positive, it follows from equation (3) that the value of Q_{10}, the van't Hoff coefficient (cf. Bělehrádek, 1935) rises with increase in T when relative Q_{O_2} is taken from the fitted curves. However, when Q_{10} is calculated from the points plotted in Figure 4, the values are somewhat irregular, as shown in Table 6. It seems reasonable to assume that this irregularity would disappear if more data were available.

c) *Qo_2-temperature-time relations.*—When relative Qo_2 is considered as a function of time as well as of temperature, the family of curves in Figure 4 shows: (1) As temperature rises, the rate of diminution of Qo_2 with time increases. Thus the difference between the 10-minute Qo_2 and the 110-minute Qo_2 increases with rise in temperature. (2) For any given temperature between 10° and 25° the slopes of the several curves decrease as time increases from 10 to 110 minutes. This indicates a decreased capacity to respond to a rise in temperature as the interval between excision of tissue and measurement of respiration lengthens. (3) As time increases, there is a decreasing diminution of Qo_2 with time (at any temperature the difference between 10-minute Qo_2 and 30-minute Qo_2 is greater than the difference between 30-minute Qo_2 and 50-minute Qo_2, and so on). This extends to the entire temperature range the same property noted in the curves of Figure 2 at 15° C.

TABLE 6

Q_{10} AS A FUNCTION OF TIME AND TEMPERATURE

Time (Minutes)	Values of Q_{10}		
	10°–15°	15°–20°	20°–25°
10	2.2	1.9	2.3
30	2.1	1.7	2.3
50	1.7	1.7	2.3
70	1.7	1.7	2.0
90	1.5	1.7	2.1
110	1.3	2.0	1.9

Since the temperature optimum for midgut-gland tissue probably is between 25° and 30°, the respiration of this organ would be profoundly depressed at typical homeotherm body-temperature levels. From this point of view, the crustacean not merely is an "incomplete" homeotherm but differs from the homeotherms qualitatively in respect to mechanisms of biological oxidation (cf. Deighton, 1933).

However, in so far as quantitative comparison is possible, the oxygen consumption of midgut gland is of the order of magnitude of that of mammalian tissue. Thus Crismon and Field (1940) found that the average Qo_2 of kidney, brain cortex, and skeletal muscle of ten adult albino rats (controls) were 17.6, 9.9, and 2.5, respectively. Assuming a Q_{10} of 2.0 for the midgut gland (Table 6) and neglecting the inhibitory effect of temperatures exceeding 25°, we find the Qo_2 of the midgut gland would be about 9.3 at 37.5°, i.e., approximately the same as rat brain cortex.

SUMMARY

1. Suitable procedures were described for measuring the oxygen consumption of the midgut gland by the Warburg manometric method.

2. The variability inherent in such procedures was assessed.

3. In a series of 61 kelp crabs (28 males and 33 females), ranging in carapace length from 1.30 to 9.68 cm. and in body weight from 0.50 to 275 gm., the mean Qo_2 was 1.73, the range 0.47–3.98.

4. The coefficients of correlation between Qo_2 and carapace length were −0.863 for

28 males, −0.815 for 33 females, and −0.845 for the series. Those for Qo_2 and log body weight were −0.863, −0.862, and −0.867, respectively. All these coefficients of correlation were significant, but the differences between them were not significant.

5. The regression coefficients of Qo_2 on either carapace length or log body weight did not differ significantly for the sexes, indicating that the Qo_2 of midgut gland does not vary with sex.

6. The Qo_2 of midgut gland rose with increase in temperature over the range 10°–25° C. The "optimum" temperature is between 25° and 30° C. The van't Hoff coefficient (Q_{10}) is close to 2 but varies with time after excision of tissue.

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CAUSE OF THE GREEN-RED COLOR CHANGE IN EUGLENA RUBRA[1]

(Four figures)

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THE genus *Euglena* includes several species which contain red-pigment granules. In *E. rubra* the apparent color of the organism can be changed from green to red by the migration of red-pigment granules from a central to peripheral or more uniformly distributed positions. Hardy (1911) in his original description of *E. rubra* described this phnenomenon but did not correlate the movement of the haematochrome granules with any extra-cellular condition. Elliot (1934) described a correlation between the presence of sunlight and the uniform scattering of the haematochrome in *Haematococcus*, and Heidt (1934) noted a similar relationship in *E. sanguinea*. During the summer of 1936 it was found that pigment migration and the ensuing color change in *E. rubra* under both natural and laboratory conditions were correlated with the presence or absence of either sunlight or artificial light (Johnson, 1939).

The purpose of the present investigation was to study the relative effectiveness of light of various wave lengths on migration of the pigment.

METHOD AND MATERIALS

E. rubra was collected from a small pond near Lake West Okoboji in northwestern Iowa. The organisms were placed in a darkroom until the red pigment had taken a central position and the macroscopic appearance of the organisms had become green. They were then placed on slides, cover slips were sealed on with vaseline, and the preparations were treated by the following methods: heated in a water bath in the dark, irradiated with infrared rays in the dark, irradiated with artificial light (500-watt tungsten-filament bulb and condenser system), and irradiated with sunlight. Corning glass color filters were used to obtain sunlight and artificial light of various wave lenghts. *Euglena* to be irradiated with sunlight were placed in small black dishes and covered with Corning glass filters.

For the visible spectrum the transmission in microns of the filters used was as follows: plain glass, 0.35–0.80 μ; No. 306, 0.40–0.80 μ; No. 352, 0.50–0.80 μ; No. 348, 0.55–0.80 μ; No. 241, 0.65–0.80 μ; No. 255, 0.75–0.80 μ; No. 554, 0.35–0.50 μ; No. 430, 0.40–0.55 μ; No. 408, 0.45–0.80 μ; and No. 511, 0.38–0.46 μ. Approximately 99 per cent of the energy transmitted by filter No. 255 was infrared, and some infrared rays were transmitted by all the other filters. In some experiments all infrared was excluded by use of Corning filter No. 398 and two inches of water. Certain filter combinations yielded relatively narrow wave-length bands. The relative energy content of the irradiating light was measured by means of a thermopile and galvanometer, and the relative energy of the artificial light was controlled by varying the distance between object and source. In some experi-

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ments the energy content of the irradiating light was the same for the various filters, and in other experiments the energy content differed in different wave bands.

Two end points were used for determining the time necessary for the color change to occur: (1) macroscopic appearance, i.e., when the treated organisms were the same color as a control group in which the haematochrome was known to be scattered peripherally; (2) microscopic appearance, i.e., when viewed under a microscope six-tenths of the organisms showed the pigment granules equally distributed peripherally. Control experi-

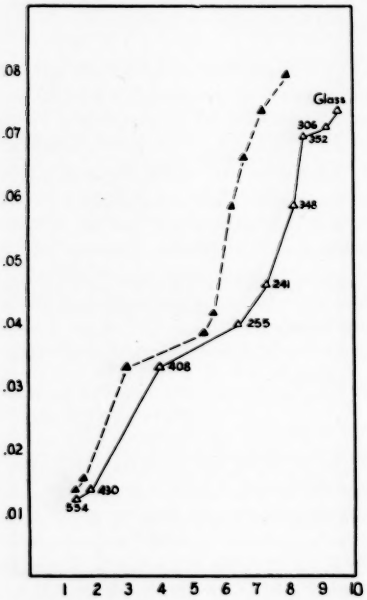

FIG. 1

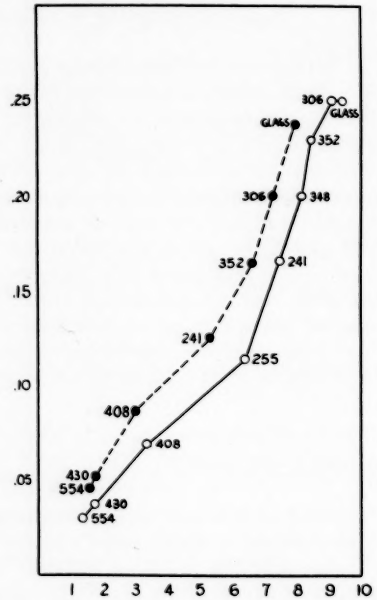

FIG. 2

FIG. 1.—The relationship between rate of migration of pigment granules and the energy of the stimulating light. Ordinate: rate of change in terms of the reciprocal of the time necessary for the pigment of six-tenths of the organisms to become distributed (microscopical observation); abscissa: energy content of the light in terms of thermopile-galvanometer deflections; solid triangles: artificial light; open triangles: sunlight. Numbers on curves denote the filters used. Temperature, about 25° C.

FIG. 2.—The relationship between rate of the color change and the energy of the stimulating light. Ordinate: reciprocal of time necessary for the macroscopic appearance to change from green to red; abscissa: energy of stimulating light in arbitrary units; solid circles: artificial light; open circles: sunlight. Numbers denote the filters used. Temperature, about 25° C.

ments were conducted in both bright light and total darkness for each series of experiments.

RESULTS

A. *The effect of high temperature and of infrared radiation.*—Organisms of *E. rubra*, kept in darkness, sealed in depression slides, and immersed in a water bath at 20° C. or

less, showed no change in the position of the colored pigment as determined microscopically. The color change was complete in those organisms maintained at 30° C. in approximately two hours. In organisms immersed in a water bath at 40° C. pigment migration was complete in approximately forty minutes. The organisms remained active and viable. Death occurred in the organisms treated similarly at 50° C.

A color change also occurred in *E. rubra* when the organisms were irradiated with infrared rays in a darkened room. The rapidity at which the pigment migration occurred was increased as the organisms were brought nearer the heat source.

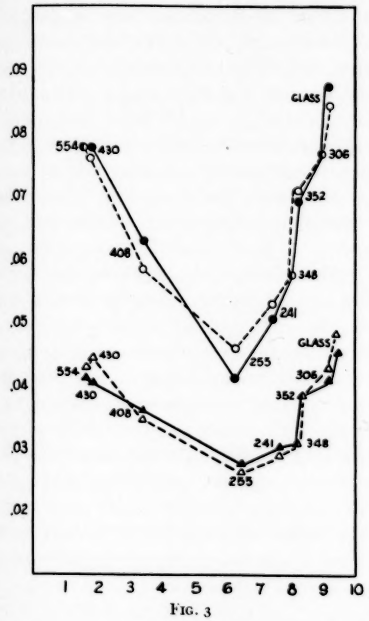

FIG. 3

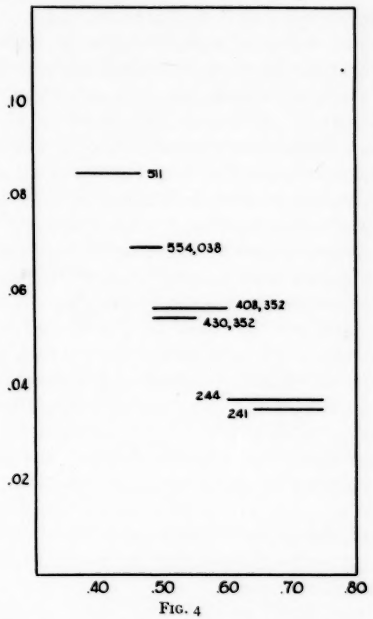

FIG. 4

FIG. 3.—The relationship between pigment migration (*lower curves*) and color change (*upper curves*) and energy of the stimulating light when heating by absorption is minimized by placing the slide in contact with ice. Symbols and numbers as in Figures 1 and 2.

FIG. 4.—Relationship between rate of color change and the wave length of the stimulating light when the energy content is the same for all wave bands.

B. *The effect of visible radiation.*—Figures 1 and 2 show the results of experiments in which filters were used and in which the organisms were maintained at the same distance from the source of energy. On the ordinate as a measure of rate is plotted the reciprocal of the time in minutes for the centrally located granules to become distributed, and on the abscissa is plotted the energy (in arbitrary units) transmitted by the various filters. The open triangles in Figure 1 denote a series in sunlight, and the solid triangles a series in artificial light, in which the end points were determined microscopically. The numbers to the right of each pair of points are those of the glass filters. The curves of Figure 2 are

exactly comparable to those of Figure 1, except that the end points were determined macroscopically. The color change occurred most rapidly in those organisms irradiated with the greatest amount of energy, irrespective of the quality of light. The curves also demonstrate that complete migration of the pigment granules is not necessary to cause an apparent color change in *E. rubra* as determined macroscopically.

Figure 3 shows the results of a series of experiments similar to the experiments plotted in Figures 1 and 2, except that all organisms are irradiated with sunlight and maintained at about 2° C. by the use of ice cubes. The ordinate and abscissa are the same as in the previous graphs. The end point was determined microscopically in the lower curves and macroscopically in the upper curves. At the same time that these experiments were performed, a control series without ice (at 25° C.) was conducted. The curve for the control series followed the same trend as those of Figures 1 and 2.

The curves of Figure 3 are quite different from those of Figures 1 and 2. The filters with which the lowest rates were obtained (Nos. 255, 241, and 348) were those which transmitted only the red end of the spectrum. The rates obtained with filters which transmitted only blue light (Nos. 554 and 430) were almost as high as those which transmitted almost the whole spectrum (clear glass and No. 306). This is clear evidence that at 2° C. blue light was much more effective in causing the color change than was red. Further evidence of this may be seen in Figure 4.

Figure 4 shows a series of experiments in which the amount of energy transmitted by the various filters was constant (at about one-half of that transmitted by clear glass). All infra-red light was excluded, and the temperature was 25 °C. On the ordinate is plotted the reciprocal of the time in minutes required for pigment distribution to occur, and on the abscissa is plotted the wave lengths in hundredths of microns. The horizontal bars show the bands of light transmitted by each filter, and the numbers near the bars denote the filters or filter combinations. The results indicate an approximately linear relationship between rate of color change and wave length. The rate of change in violet light (filter No. 511) was about two and one-half times as high as in deep-red light (filter No. 241). Preliminary experiments in which all the infrared was not excluded gave essentially the same results as those shown in Figure 2, except that the difference in rate between the longer and the shorter wave lengths was not so great.

DISCUSSION

The present experiments demonstrate that the green-red color change in *E. rubra* can be induced by several methods. The change can be brought about by heating to 30°–40° C. and by infrared irradiation. Since the temperature of the cells is raised by both of these procedures, we may consider the effects to be strictly comparable. The results shown in Figures 1 and 2 may also be considered to be caused at least partially by local heating, since the organisms were exposed to very bright light and absorption by the cells was undoubtedly considerable. On this assumption the results are what should be expected, in that the rate of migration was roughly proportional to the energy input, regardless of the wave length of the stimulating light.

The data of Figure 3 demonstrate that, when heating of the cell is reduced to a minimum (by contact with an ice cube), the wave length of the incident light is very important and the change occurs much more rapidly when the blue end of the spectrum is present. This is further substantiated by the data of Figure 4, in which it is demonstrated

that, in response to stimulation by bands of wave lengths, the blue end of the spectrum is much more effective than the red. Since the experiments of this series were performed at 25° C., the data demonstrate that the greater effectiveness of blue light shown in Figure 3 is not limited to experiments at low temperatures. During the experiments of Figure 4, local heating of the organisms probably occurred to a slight degree. However, since all infrared was excluded and the visible transmitted bands were considerably narrower, the total energy content of the stimulating light was much lower than in previous experiments and heating was probably slight.

The function of the haematochrome and the mechanism of migration are largely a matter of conjecture. Since the pigment migrates to a position which shields the chromatophores from very bright light, it may be considered to have a protective action. When the granules are perhipherally distributed, much of the blue end of the spectrum may be removed by absorption and the red end by reflection from the surface of the granules. The analysis by Tischer (1936) of the pigment of *E. heliorubescens* demonstrated that the principal component of haematochrome is a tetraketo-β-carotin (euglenarhodon), which has a high absorption in the blue region of the spectrum. Preliminary observations of the present authors made with a microspectroscope indicate that the absorption of the pigment of *E. rubra* is probably similar to that of *E. heliorubescens*. The effectiveness of the different wave bands in causing migration approximates the absorption curve of the pigment, but the mechanism by which migration is effected is entirely unknown.

If haematochrome were to exert a protective action on the chloroplasts, this phenomenon might explain the geographical and temporal distribution of *E. rubra*. Hardy (1911) found this organism when the air temperature was 90°–100° F. The present authors have observed the organism in northwestern Iowa every summer since 1936. In every case the greatest numbers occurred during very hot weather, especially when the temperature had been consistently above 90° F. for a week or more and the wind velocity low. With one exception the organism was confined to shallow ponds (2–3 feet in depth), usually those to which cattle or hogs had access. The exceptional case was Little Miller's Bay of Lake West Okoboji in the summer of 1936, during a week when air temperatures were often above 97° F., wind velocity was negligible, and clouds were absent. During the day the water temperature was 35°–45° C. in the shallower portions, and *E. rubra* formed a bright red scum on the surface. It seems possible that it is the presence of haematochrome which permits *E. rubra* to thrive under such conditions. During cooler weather the organisms must be very few in number, and they might exist in a green phase which contains very few haematochrome granules.

SUMMARY

1. It was found that the green-red color change in *E. rubra* could be induced by heating to 30°–40° C. and by irradiation with infrared rays or visible light.

2. The effectiveness of various wave bands in causing the green-red color change was tested.

3. It was found that, when the intensity is approximately that of bright sunlight and when heating of the cells is not prevented, the effectiveness of the wave band used is a function of the energy of the stimulating light, irrespective of the wave length.

4. When the temperature of the preparation is maintained at about 2° C., wave bands which include only the blue end of the spectrum are almost as effective as the whole

spectrum, irrespective of the relative energy transmitted in each case, in the intensity range studied.

5. When, at 25° C., heating of the preparation is slight because of low energy content of the stimulating light, the wave bands which include only the blue end of the spectrum are much more effective than those which include only the red end.

6. The possible significance of the presence of haematochrome in *E. rubra* is discussed.

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INCOMPLETE PROTEINS AS NITROGEN SOURCES, AND THEIR RELATION TO VITAMIN REQUIREMENTS IN COLPIDIUM CAMPYLUM

(Seven figures)

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OBSERVATIONS on various ciliates have indicated that the nitrogen requirements of these organisms include proteins or their more complex cleavage products. The food requirements of several species in pure cultures (Lwoff, 1932; Elliott, 1933; Johnson, 1935; Loefer, 1934, 1936) are satisfied by one or more inorganic salts and a suitable commercial peptone, while other ciliates have been grown only in more complex media (Glaser and Coria, 1933, 1935). For certain of these ciliates, proteinases have been reported (Lwoff and Roukhelman, 1929; Elliott, 1933; Lawrie, 1937), and digestion of such substances as casein, gelatin, and egg albumin has been observed. Nevertheless, growth of several species has been most abundant in media containing partially digested proteins, and in some cases the amount of growth has appeared to be correlated with the concentration of amino acid nitrogen in a given "peptone." Hence it appears that the most suitable media for several of the free-living ciliates are those which contain not only the more complex protein derivatives but also readily utilizable amino acids in favorable concentrations.

That such a general statement may be subject to qualification has been suggested by reports that growth of several ciliates is not supported by "incomplete" proteins or their derivatives, which are deficient in some of the so-called essential amino acids (Lwoff, 1932; Lwoff and Lwoff, 1937; Elliott, 1935; Hall and Elliott, 1935). The recent observations on growth factors (vitamins) in bacteria and Protozoa suggested the possibility that growth of ciliates in such "incomplete" protein media might be induced by the addition of various substances known to accelerate metabolic activities. The writer became interested in the question during the course of observations on pimelic acid (Hall, 1939*a*), which was found to accelerate growth of *C. campylum* in a peptone medium. Further tests were carried out with a gelatin medium supposedly inadequate for growth of this species. The observed effects—which included fairly good growth with pimelic acid—suggested the use of gelatin media in the serial-transfer technique, as well as similar tests of silk-peptone media. Results obtained with these media necessitated the consideration of vitamin requirements in *C. campylum*.

MATERIAL AND METHODS

The bacteria-free strain of *C. campylum*, used in the experiments described below, was obtained from Dr. T. L. Jahn and has been maintained in our laboratory for several years in various peptone media (Hall, 1937). Purified gelatin (Difco), de-ashed gelatin (Eastman), La Roche silk-peptone (Hoffmann-La Roche), dextrose (Difco; Baker's "Analyzed"), thiamin hydrochloride (Merck's "Betabion"), riboflavin (General Bio-

chemicals) and pimelic acid (S.M.A. Corporation) were added to the different media, as specifically indicated in each case. All inorganic salts were "analyzed reagent" quality (Baker, Mallinckrodt, or Merck). Distilled water was redistilled in Pyrex glass before use.

The serial-transfer technique was used in testing each medium. In this procedure the tube cultures in Transfer 1 of a given series are inoculated from a suitable stock culture; the tubes of Transfer 2 are inoculated from a tube of Transfer 1 after incubation, and so on in subsequent transfers. This technique effects a serial dilution of the materials carried over from the stock culture, and growth of the ciliates eventually becomes dependent entirely upon the food present in the experimental medium. Tubes were fixed for counting immediately after inoculation and again at the end of incubation; the corresponding initial and final counts (numbers of ciliates per cubic centimeter) furnished an index to the amount of growth in each transfer. The final counts for the various series are recorded graphically in Figures 1–7. Our method of counting has been described elsewhere (Hall, Johnson, and Loefer, 1935).

In each series the medium was tubed in either 9.0- or 10.0-cc. amounts and then sterilized in the autoclave at 122° C. for 20 minutes. Initial pH was determined after inoculation, and final pH after incubation of each transfer. Since the changes in pH were found to be minor ones in most cases, only the initial pH readings are recorded below. A LaMotte roulette comparator was used in all pH determinations.

GROWTH IN SILK-PEPTONE MEDIA

Since silk-peptone, an "incomplete" protein derivative, had failed to support growth of *Glaucoma piriformis* (Lwoff and Lwoff, 1937), this peptone was tested on *C. campylum*, as described.

SERIES I–IV

The basic medium in these series contained silk-peptone (1.0 per cent) and KH_2PO_4 (0.1 per cent). In Series I, pimelic acid (1×10^{-6} gm. per cubic centimeter and Difco dextrose (0.25 per cent) were added to the medium; in Series II, dextrose only; in Series III, pimelic acid was added in Transfers 1–8 but not in subsequent transfers; in Series IV, only the basic medium was used. The initial pH was 4.8 for the first transfer in all series; in later transfers the initial pH varied from 5.1 to 5.9. In Series I and II, tubes of the first transfer were inoculated from a stock culture in casein-peptone medium; in Series III and IV, from a strain of *C. campylum* in gelatin medium (Ser. XVI, Transfer 8). In order to increase the rate of dilution in later transfers, the following procedure was adopted. After incubation of the cultures in Transfer 1, a measured quantity of culture material was transferred to a tube containing the experimental medium. The latter was then used for inoculating the tubes of Transfer 2. This procedure was repeated in all except Transfers 7, 9, and 10, in which a tube of the preceding transfer was used as the direct source of inocula. In this way the protein derivatives carried over from the stock culture were reduced to less than 3×10^{-20} gm. per cubic centimeter in Transfer 10.

Series I.—Successive initial counts were 183, 315, 201, 200, 138, 185, 2,301, 110, 4,775, 4,502. The density of population after incubation ranged from about 21,000 to approximately 59,000 per cubic centimeter (Fig. 1). The lowest density (Transfer 8) was observed after an incubation period of 48 hours; the higher populations in the other transfers are to be correlated with incubation periods of 4–9 days. The series was discontinued after the eleventh transfer.

Series II.—Initial counts in successive transfers were as follows: 197, 193, 151, 135, 105, 55, 2,695, 246, 4,961, 4,038. The density of population (Fig. 1) in each transfer was similar to that observed in Series I.

Series III.—Initial counts for the first 10 transfers were 830, 330, 158, 438, 475, 143, 80, 1,118, 3,685, 3,065. The final populations (Fig. 1) approximated those observed in corresponding transfers of Series I and II. This series was continued for 27 transfers through a period of more than 16 months. In general, growth was fairly consistent, and, with a suitable period of incubation, a population of 40,000–60,000 per cubic centimeter

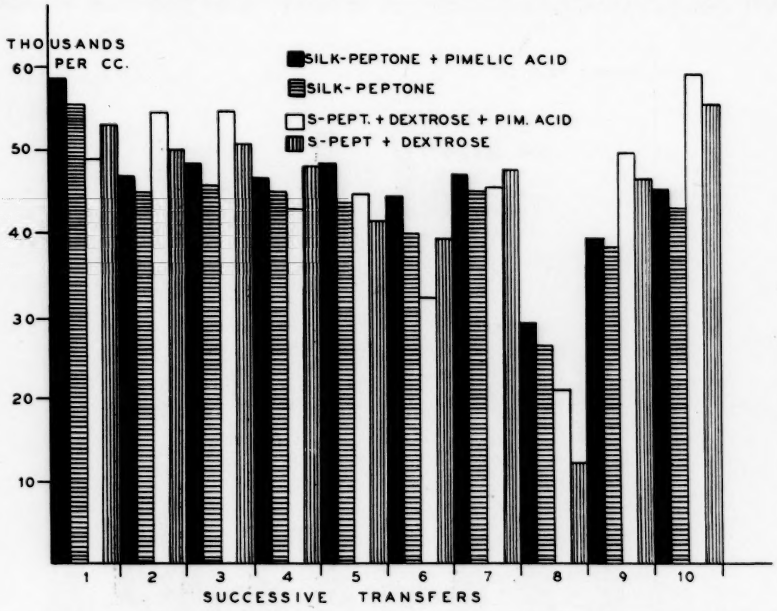

FIG. 1.—Growth of *C. campylum* in silk-peptone media. Series I, silk-peptone, dextrose, and pimelic acid; Series II, silk-peptone and dextrose; Series III, silk-peptone and pimelic acid; Series IV, silk-peptone alone.

may be expected in silk-peptone medium. However, such a peak is maintained for only a few days. In this series the pimelic acid from Transfer 8 was diluted to the order of 1.7×10^{-30} in Transfer 27. Hence, it is obvious that silk-peptone, without any added growth stimulant, is an adequate food for *C. campylum*.

Series IV.—In this series the successive initial counts were closely comparable to those listed for Series III, and the corresponding densities of population (Fig. 1) were likewise similar to those noted in the preceding series. Series IV was maintained for 15 transfers and then discontinued. The results, as in Series III, demonstrate that silk-peptone alone is adequate for growth of *C. campylum*.

Since the Lwoffs (1937) have maintained that La Roche silk-peptone contains no thiamin (vitamin B_1), it would thus seem that *C. campylum*, being capable of growth in this peptone medium, does not require thiamin as a growth factor. However, thiamin

appears to be essential for growth of *Glaucoma piriformis* (Lwoff and Lwoff, 1937, 1938) and *C. striatum* (Elliott, 1937, 1939), and it seemed unlikely that *C. campylum* would differ so markedly from these two related ciliates. This question, accordingly, was considered in Series V–VIII.

Series V–VIII.—The basic medium contained de-thiaminized silk-peptone (1.0 per cent) and KH_2PO_4 (0.1 per cent). A solution of peptone was heated at pH 9.6–9.8 for 20–30 minutes, according to the method followed by the Lwoffs (1938) in destroying thiamin. Then, KH_2PO_4 was added and the pH lowered by the addition of HCl. Series V–VII were inoculated from Transfer 8 of Series IV; Series VIII, from Transfer 1 of

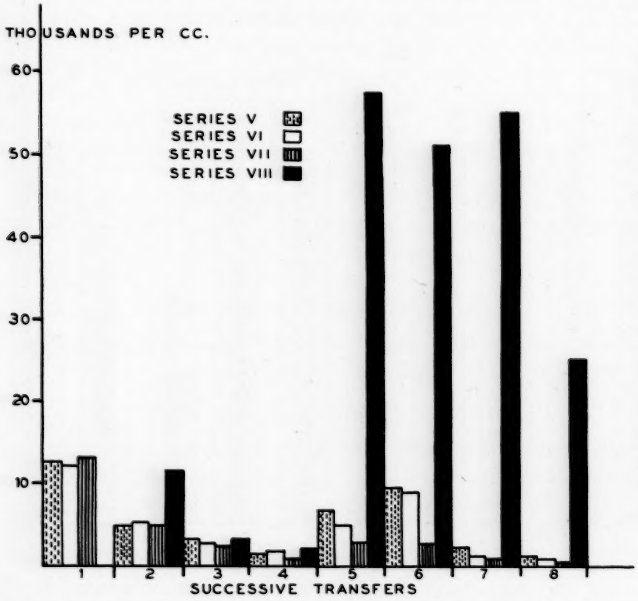

FIG. 2.—Growth of *C. campylum* in de-thiaminized silk-peptone. Series V, de-thiaminized silk-peptone; Series VI, pimelic acid added; Series VII, dextrose added; Series VIII, thiamin added.

Series V. Series V served as a control, while pimelic acid (1×10^{-6} gm. per cubic centimeter), Baker's dextrose (0.25 per cent), and thiamin (1×10^{-6} M) were added to the medium in Series VI, VII, and VIII, respectively. The initial pH ranged from 6.1 to 7.5 and, with one exception (Ser. VII, Transfer 6), was the same in the corresponding transfers of all series. Successive incubation periods were 5, 7, 8, 8, 7, 7, 9, and 13 days. The technique of inoculation was such that the concentration of untreated silk-peptone, introduced in Transfer 1, was reduced to less than 6×10^{-10} gm. per cubic centimeter in the last transfer. The density of population (Fig. 2), except in the later transfers of Series VIII with thiamin, was always much lower than in normal silk-peptone medium. Growth in the control series and in the media containing dextrose (Ser. VI) and pimelic acid (Ser. VII) was so slow that these strains could not have been continued for more than

one or two additional transfers. The results thus indicate that thiamin is essential for normal growth of *C. campylum* and also that La Roche silk-peptone must contain thiamin, contrary to the conclusion of the Lwoffs.

The variable results obtained with thiamin in different transfers (Fig. 2) require explanation. An old thiamin solution, which was used in Transfer 2, had already been sterilized in the autoclave several times; this solution was sterilized again in each case after use in Transfers 2 and 3. A fresh solution was made up for Transfer 5 and sterilized afterward; this same solution was used and sterilized subsequently in Transfers 6 and 7. By the eighth transfer, the activity of this solution had been reduced appreciably. In

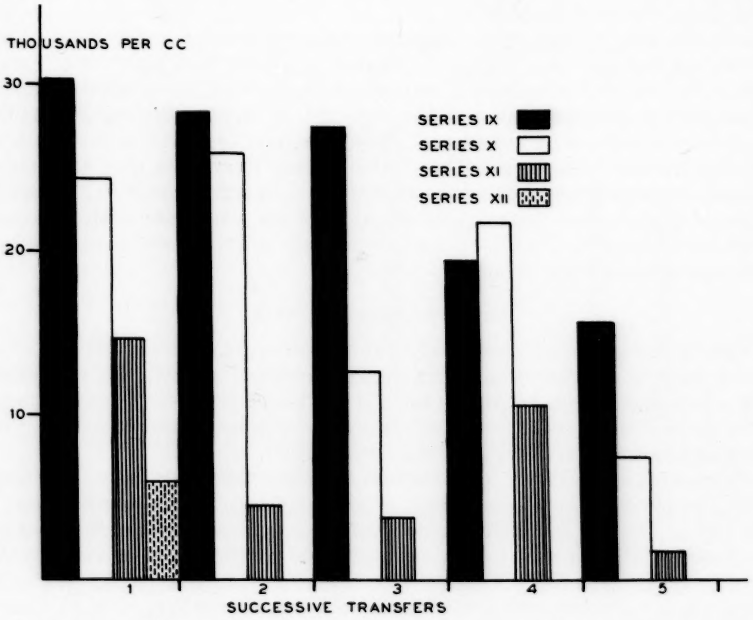

FIG. 3.—Growth of *C. campylum* in de-thiaminized silk-peptone. Series IX, peptone treated for 20 minutes, thiamin added; Series X, peptone treated for 90 minutes, thiamin added; Series XI, peptone treated for 20 minutes, no added thiamin; Series XII, peptone treated for 90 minutes, no added thiamin.

addition the solution of silk-peptone was heated at pH 9.6–9.8 for different intervals in the different transfers of Series V–VIII.

In view of these irregular results, the effects of heating silk-peptone in alkaline solution for different lengths of time were determined in Series IX–XII. In Series IX and XI a 2.0 per cent peptone solution was de-thiaminized as in Series V–VIII, but the solution was heated for exactly 20 minutes at 122° C. In Series X and XII a similar solution of silk-peptone was heated for 90 minutes. After heating, each peptone solution was added to an equal volume of salt solution, so that the final medium contained silk-peptone (1.0 per cent) in the following mixture of salts: $MgSO_4 \cdot 7H_2O$ (0.02 per cent),

KH_2PO_4 (0.02 per cent), $CaCl_2 \cdot 2H_2O$ (0.01 per cent), $FeCl_3 \cdot 6H_2O$ (0.00025 per cent) $MnCl_24 \cdot H_2O$ (0.000001 per cent). The pH was reduced to a satisfactory level, and each medium was tubed and sterilized at 122° C. for 20 minutes. Before sterilization, each tube in Series IX and X received 1.0 cc. of thiamin solution (1×10^{-5} M); each tube in Series XI and XII, a corresponding volume of distilled water.

All four series were started from Transfer 15 of Series IV, and the initial counts (660–925 per cubic centimeter) were comparable in the first transfers of the four series. The initial pH was 7.3 in Transfer 1, and 6.7–7.1 in subsequent transfers. Periods of incubation were 8, 10, 9, 10, and 10 days, respectively. The technique of inoculation was such that the untreated peptone introduced in Transfer 1 was reduced to less than 5×10^{-8} gm. per cubic centimeter in Transfer 5.

The results (Fig. 3) confirm the previous conclusion that La Roche silk-peptone contains thiamin and that this vitamin is essential to growth of *C. campylum*. Series IX–XII show, further, that the effects of heating silk-peptone in alkaline solution are dependent upon time of treatment, as would be expected. Prolonged heating renders the medium entirely unsuitable for growth of *C. campylum*, since Series XII showed growth only in the first transfer. On the other hand, the medium treated for only 20 minutes still supported very slow growth of the ciliates (Ser. XI). In either case (Sers. IX and X) the addition of thiamin was distinctly beneficial, and the results obviously indicate that thiamin, normally present in silk-peptone, is partially or completely inactivated by heating the peptone in alkaline solution.

GROWTH IN GELATIN MEDIA

In view of the earlier failures (Lwoff, 1932; Elliott, 1935; Hall and Elliott, 1935), attempts to grow *C. campylum* in gelatin media were based at first upon the addition of growth stimulants to such media. Series XIII–XVI were designed to test the possibility that pimelic acid might serve as a growth factor, since an accelerative effect on growth of *C. campylum* had already been observed (Hall, 1939*a*).

In Series XIII and XIV the basic medium contained Difco gelatin (1.5 per cent) and KH_2PO_4 (0.2 per cent) in distilled water; in Series XV and XVI this medium was modified by the addition of Difco dextrose (0.5 per cent in Transfers 1–4; 0.25 per cent in later transfers). Pimelic acid (1×10^{-6} gm. per cubic centimeter) was added to the medium in Series XIV and XVI. The first transfers in the four series were inoculated from a stock culture in casein-peptone medium, and the initial counts (433–82 ciliates per cubic centimeter) thus were comparable. In all transfers each tube received a 0.5-cc. inoculum. The initial pH ranged from 5.0 to 5.5 in different transfers. Incubation periods for Transfers 1–4 were 11, 10, 10, and 13 days, respectively; later transfers (Sers. XIV and XVI) were incubated 7–15 days.

The results (Fig. 4) indicate that the addition of pimelic acid was advantageous. Thus, the ciliates were maintained for five transfers in Series XIV and for nine in Series XVI, while the strains in the corresponding media without pimelic acid survived for only two or three transfers. Through serial dilution, the peptone carried over from the stock culture was reduced to less than 4.5×10^{-14} gm. per cubic centimeter in Transfer 9 of Series XVI. Hence, it seemed probable that *C. campylum* is capable of continued growth in a medium containing Difco gelatin, dextrose, and pimelic acid.

These results suggested that pimelic acid may serve as a growth factor (vitamin), as reported in a preliminary note (Hall, 1938*b*). Later observations, described below, have

indicated that pimelic acid is not essential for growth of *C. campylum* and is to be regarded as a growth stimulant rather than a true growth factor for this organism.

At this point it was decided to test Difco gelatin (1.5 per cent) in a solution of certain inorganic salts (the same as used in Series IX–XII), since this combination is moderately satisfactory for *Euglena anabaena* (Hall, 1938*a*). In Series XVII this medium was used as a control, while pimelic acid (1×10^{-6} gm. per cubic centimeter) was added in Series XVIII. Both series were started from a stock culture in casein-peptone.

In Series XVII successive initial counts were 420, 643, 204, 1,011, 1,158. The initial pH ranged from 6.1 to 6.9 in different transfers; incubation periods ranged from 7 to 12 days. In Series XVIII the initial counts were 485, 1,209, 1,138, 1,114, 1,283. Incubation

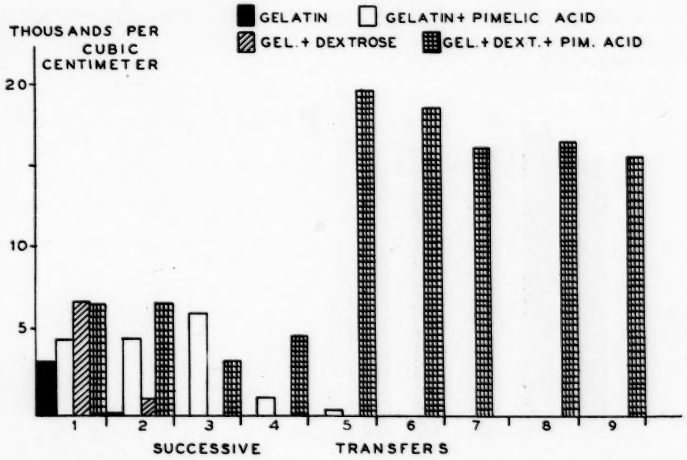

FIG. 4.—Growth of *C. campylum* in gelatin media. Series XIII, gelatin alone; Series XIV, gelatin and pimelic acid; Series XV, gelatin and dextrose; Series XVI, gelatin, dextrose, and pimelic acid.

periods and initial pH were the same as in Series XVII. Moderate growth occurred in each transfer and, except for Transfer 2, the final populations were comparable in the two series (Fig. 5). The results suggested that pimelic acid is not essential for growth of *C. campylum* in a medium containing Difco gelatin and several inorganic salts. This conclusion was confirmed in Series XIX.

Series XIX, which was essentially a duplicate of Series XVII, was started for direct comparison with Series XXIV–XXVII, as described below. Tubes of the first transfer were inoculated from a strain of *C. campylum* in silk-peptone medium (Ser. I, Transfer 11). The initial pH has ranged from 5.5 to 7.1 in different transfers. Incubation periods for the first 10 transfers were 4, 8, 8, 7, 9, 9, 13, 8, 10, and 10 days, respectively. Fairly good growth (Fig. 6) has occurred in each transfer, and the population has ranged from approximately 11,000 to 25,000 per cubic centimeter. This strain is now in Transfer 29 and has been maintained for approximately 21 months.

The results obtained in Series XVII and XIX thus demonstrate that Difco gelatin and several inorganic salts constitute a satisfactory medium in which *C. campylum* may

be maintained indefinitely by means of the serial-transfer technique. Hence it may be concluded that this type of gelatin contains all the essential organic foods as well as any growth factors which may be necessary for this ciliate. However, it is possible that the concentration of some of these essential substances does not approach the optimum, since the density of population in gelatin medium is much lower than that observed in our stock-culture media or even in silk-peptone media.

De-ashed gelatin, known to be more highly purified than Difco gelatin, was next tested as a source of food for *C. campylum*. It was expected that this type of gelatin would be more deficient than Difco gelatin with respect to mineral constituents, and the results of several preliminary series (XX–XXIII) confirmed this expectation.

In Series XX–XXIII the basic medium contained de-ashed gelatin (1.5 per cent). Difco dextrose (0.1 per cent), and KH_2PO_4 (0.1 per cent). This medium was used as the

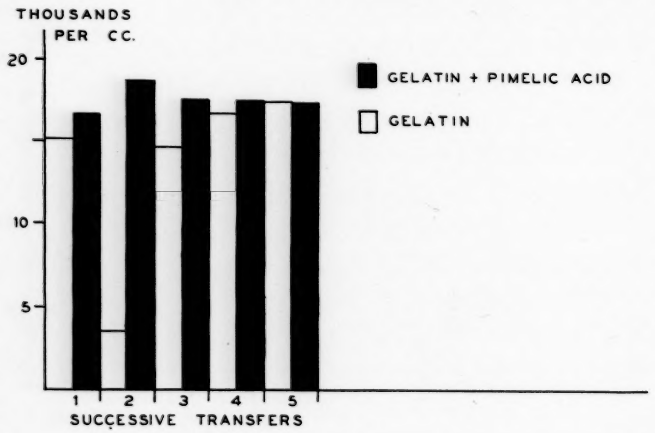

FIG. 5.—Growth of *C. campylum* in gelatin media containing several inorganic salts. Series XVII, gelatin control; Series XVIII, gelatin and pimelic acid.

control in Series XX. Suberic, adipic, or azelaic acid, 1×10^{-6} gm. per cubic centimeter, was added to the medium in each of the other series. All series were inoculated with a strain of *C. campylum* in Difco gelatin. In each case the first transfer showed no significant growth, and there was a distinct decrease in number of ciliates in the second transfer. The results indicated that a medium containing de-ashed gelatin as the nitrogen source will not support growth of *C. campylum*.

In Series XXIV–XXVII, de-ashed gelatin (1.5 per cent) was added to the salt solution which was used in Series XVII–XIX. This basic medium served as the control in Series XXIV. Pimelic acid (1×10^{-6} gm. per cubic centimeter) was added to the medium in Series XXV; thiamin (1×10^{-6} M), in Series XXVI; Baker's dextrose (0.25 per cent), in Series XXVII. All four series were inoculated from Series I (Transfer 11) and were incubated concurrently with Series XIX. The initial pH ranged from 5.7 to 6.6 in different transfers. The inocula in Transfer 1 were fairly heavy, and the initial counts ranged from 4,151 to 4,420 for the different series. A steady decrease in density of population

(Fig. 6) was noted in successive transfers, and no ciliates were seen in the eighth and subsequent transfers in these series.

The favorable effects of added thiamin in Series XXVI suggested that de-ashed gelatin is probably deficient in this vitamin. However, the density of population decreased steadily even with added thiamin, and it was suspected that this type of gelatin might be deficient in more than one growth factor. This possibility was considered in Series XXVIII–XXXI.

Series XXVIII–XXXI.—In these series the basic medium contained de-ashed gelatin (1.0 per cent) in the same salt solution as used in Series XXIV–XXVII. This medium

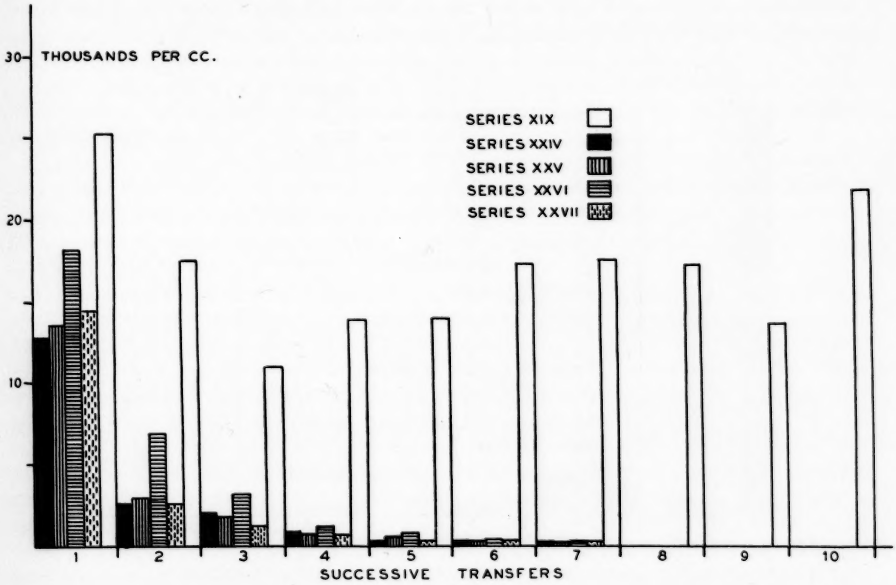

FIG. 6.—Growth of *C. campylum* in de-ashed gelatin media and in a Difco gelatin medium. Series XIX, Difco gelatin; Series XXIV, de-ashed gelatin; Series XXV, de-ashed gelatin and pimelic acid; Series XXVI, de-ashed gelatin and thiamin; Series XXVII, de-ashed gelatin and dextrose.

served as the control in Series XXVIII. The following growth factors were added in the other series: thiamin (1×10^{-6} gm. per cubic centimeter), Series XXIX; riboflavin (1×10^{-6} in Transfer 1; 1×10^{-7} gm. per cubic centimeter in later transfers), Series XXX; both thiamin and riboflavin, Series XXXI. All series were started from a strain of *C. campylum* in Difco gelatin (Ser. XIX, Transfer 21), and the initial counts for the first transfer ranged from 275 to 1,223 ciliates per cubic centimeter in the different series. Initial pH varied from 6.0 to 6.3. The four series were run concurrently in an incubator at 25° C., the incubation periods ranging from 7 to 17 days for Transfers 1–5 and from 18 to 40 days in later transfers (Ser. XXXI).

Growth in the control series (Ser. XXVIII) was observed only in the first transfer

(Fig. 7); with added thiamin (Ser. XXIX), growth occurred in the first and second transfers, but not thereafter; with added riboflavin (Ser. XXX), the results were no better than in the control medium. With both thiamin and riboflavin (Ser. XXXI), however, the ciliates have continued to grow. Final populations for the first five transfers of Series XXXI are recorded in Figure 7. The final count for Transfer 10 was 1,493 per cubic centimeter, almost the same as in Transfer 5. The strain is now in the twelfth transfer and is more than 9 months old. These results indicate that de-ashed gelatin is deficient in both thiamin and riboflavin and also that the ciliate, *C. campylum*, requires both thiamin (vitamin B_1) and riboflavin (B_2) as growth factors. Upon addition of these

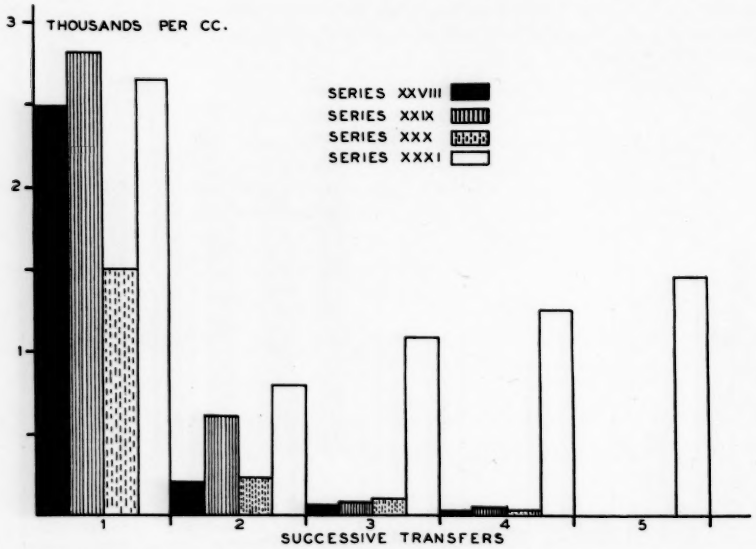

FIG. 7.—Growth of *C. campylum* in de-ashed gelatin media. Series XXVIII, de-ashed gelatin; Series XXIX, de-ashed gelatin with thiamin added; Series XXX, de-ashed gelatin with riboflavin added; Series XXXI, de-ashed gelatin with both thiamin and riboflavin.

two vitamins, a medium containing de-ashed gelatin as the only organic food becomes adequate for continued slow growth of *C. campylum*.

DISCUSSION

The observations recorded above indicate that both thiamin and riboflavin (vitamins B_1 and B_2, respectively) are essential in metabolism of the ciliate, *C. campylum*. Although a need for thiamin has been reported previously in two other ciliates—*C. striatum* (Elliott, 1937, 1939) and *Glaucoma piriformis* (Lwoff and Lwoff, 1937, 1938)—this appears to be the first instance in which riboflavin has been found necessary for growth of a protozoön. These vitamin requirements are related directly to the question of incomplete proteins as sources of food for ciliates.

It is now obvious that, without added growth factors, La Roche silk-peptone and a commercial preparation of gelatin (Difco) will support growth of *C. campylum*. With added thiamin and riboflavin, de-ashed gelatin (Eastman) also will support growth of this ciliate. These results make it necessary to discard earlier beliefs (e.g., Lwoff, 1932) that incomplete proteins, because of their deficiencies in essential amino acids, are inadequate foods for ciliates. The observations on *C. campylum* permit the following alternative conclusions: that such "incomplete" proteins actually contain the essential amino acids generally believed to be missing or that these so-called essential amino acids are not actually essential to growth of the ciliate tested. If the accuracy of various organic analyses is taken for granted, the first possibility must be dismissed. Acceptance of the second conclusion would imply that certain amino acids (e.g., tryptophane, possibly histidine and threonine) are not essential in the metabolism of *C. campylum* or else that these amino acids can be synthesized by the ciliate. It would necessarily follow that the nitrogen requirements of certain ciliates may be comparatively simple, contrary to Lwoff's (1932) opinion that the ciliates are strictly comparable to the mammals in their nitrogen requirements.

Comparison of the results obtained with three types of incomplete proteins shows wide differences in the density of population attained by *C. campylum*. For instance, de-ashed gelatin, even with added thiamin and riboflavin, is still a poor medium as compared with silk-peptone. Hence, it seems likely that this type of gelatin is deficient in other respects, as well as in the content of thiamin and riboflavin.

The demonstration that La Roche silk-peptone contains thiamin has a bearing on the reported synthesis of this vitamin by *Acanthamoeba castellanii* (Lwoff, 1938). *Glaucoma piriformis*, which failed to grow in silk-peptone media (Lwoff and Lwoff, 1937), did grow upon inoculation into thriving cultures of *A. castellanii* in silk-peptone medium. These results were assumed to indicate that the amoeba had synthesized thiamin, which in turn supported growth of *G. piriformis*. This conclusion was based upon the belief (Lwoff and Lwoff, 1937) that La Roche silk-peptone contains no thiamin. Since thiamin has now been demonstrated in this peptone, there seems to be no sound evidence for Lwoff's conclusion that thiamin is synthesized by *A. castellanii*. Such experiments obviously must be carried out in test media which are demonstrably deficient in the growth factor under consideration, and this criterion cannot be applied to Lwoff's (1938) experiments. Actually, the inability of the Lwoff's (1937) to grow *G. piriformis* in silk-peptone media may be the result of their consistent use of small inocula and relatively short incubation periods. Under such conditions it may be impossible for growth of ciliates to keep pace with the rate of dilution in the serial-transfer technique.

This question of the synthesis of thiamin is a fundamental one, since it is related to apparently basic differences between the plantlike flagellates and other Protozoa. Recent observations have shown that several Phytomastigophora, both colorless (Mast and Pace, 1933; Schoenborn, 1940) and chlorophyll-bearing (Dusi, 1933*a*, 1933*b*; Hall, 1938*a*, 1939*b*; Hall and Schoenborn, 1939), can be grown in inorganic salt solutions or in such media to which acetate or a similar carbon source has been added. Such media contain no vitamins. Hence, flagellates which grow in these media either do not require thiamin at all, or else they are able to synthesize this vitamin from the constituents of the medium. Although the synthesis of thiamin by a plantlike flagellate has not yet been demonstrated, it has been assumed that such organisms do effect this synthesis. On the other hand, there is no doubt that the Zoömastigophora and other Protozoa, which have

been investigated, all require media containing thiamin. Furthermore, in the absence of any definite evidence to the contrary, it must be assumed that these Protozoa are unable to synthesize thiamin. The physiological peculiarities responsible for such basic differences in vitamin requirements must remain matters for speculation until more has been learned about essential food requirements of the higher Protozoa and about the fundamental metabolic processes of both plantlike flagellates and other Protozoa.

SUMMARY

Thiamin (vitamin B_1) and riboflavin (vitamin B_2) seem to be essential for growth of *C. campylum* in a de-ashed gelatin medium.

A less highly purified gelatin, in a solution of inorganic salts, supported growth of this ciliate without added growth factors.

Silk-peptone, without added vitamins, likewise was an adequate source of food. However, prolonged heating in strongly alkaline solution rendered this peptone unsatisfactory for *C. campylum*. The addition of thiamin counteracted such effects—at least, to the extent that the ciliates continued to grow with the added vitamin.

The bearing of these results on the nitrogen requirements of ciliates is discussed.

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